SCREENING FOR VIRAL INFECTIONS IN MUSCLE BIOPSIES OF PATIENTS WITH ACUTE RHABDOMYOLYSIS AND INFLAMMATORY MYOPATHY

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ABBREVIATION

AGE	Agarose gel electrophoresis
ARF	Acute renal failure
ATP	Adenosine triphosphate
B actin	Beta actin
BUN	Blood urea nitrogen
cDNA	Complementary deoxyribonucleic acid
CK	Creatinine Kinase
CMV	Cytomegalovirus
CPK	Creatine phosphokinase
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EMG	Electromyography
EV	Enterovirus
EV71	Enterovirus 71
HSV	Herpes simplex virus
IBM	Inclusion body myositis
IHC	Immunohistochemistry
ISH	In-situ hybridization
KCl	Potassium chloride

LDH	Lactic dehydrogenase
MDCK	Madin-Darby Canine Kidney
$MgCl_2$	Magnesium chloride
MMLV	Moloney Murine Leukemia Virus
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
$(NH)_2SO_4$	Ammonium sulphate
OCT	Optimal cutting temperature
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium doedycyl sulphate
SGOT	Serum glutamic oxaloacetic transaminase
TBE	Tris-Borate EDTA
TBS	Tris buffer solution
T_m	Melting temperature
UMMC	University Malaya Medical Centre
UV	Ultraviolet
VZV	Vericella zoster virus
WNV	West-nile virus

ABSTRACT

ABSTRACT

Rhabdomyolysis is the dissolution of skeletal muscles that causes the release of toxic intracellular contents into an individual's circulatory system. Inflammatory myopathy, which consists of polymyositis, dermatomyositis and inclusion body myositis is also a form of muscle weakness which is milder than rhabdomyolysis in general. To date, the association between rhabdomyolysis and viral infection had been documented in numerous literatures. However, direct association in between 2 different inflammatory myopathies (polymyositis and inclusion body myositis) with viral infection had not been shown so far. None of such studies had been conducted in Malaysia so far, thus prompted the interest to investigate the association of these 2 muscular diseases with viral infection in a Malaysian population. In this study, 13 cases (5 acute rhabdomyolysis cases and 8 inflammatory myopathy cases) were selected based on their respective anatomic pathology reports which were suggestive of viral infection. RNA was extracted by using a combination of TRIzol ® Reagent and Proteinase K for the digestion of muscle while DNA were extracted using conventional Phenol-chloroform method. These samples were later subjected to 2 step RT-PCR, with random hexamer used in the RT steps to produce cDNA, followed by PCR amplification. Positive control extracted from cultures of viral isolates, negative control using double-distilled MilliQ water as template and internal control using Beta actin primers were included. 3 types of viral degenerate primers (Enteroviruses, Flaviviruses, Herpesviruses) and another 2 sets of viral primers (Influenza A and Influenza B)

were used to screen for the presence of virus, if any, from these 5 different types of viruses from 4 families. Results have shown that all cases were negative except for 4 inflammatory myopathy cases screened for Enterovirus and another 3 inflammatory myopathy cases screened for Influenza A where there were presence of multiple non specific bands. One of these suspected positive cases (P03/3-suspected EV positive) were sent for DNA sequencing and the result showed that the amplified region was not a viral genome from the EV genus, thus concluded that the presence of multiple bands for all the suspected positive cases were non specific bands. Immunohistochemistry (IHC) was also performed on the similar case and the result was negative. Larger sample size is required in future in order to investigate the association between viral infection and these muscular diseases.

CHAPTER 1
INTRODUCTION

INTRODUCTION

1.1 Historical Background of Rhabdomyolysis

According to Guis *et. al*, the first historical reference to rhabdomyolysis is probably a passage in the Bible (Old Testament, Book of Numbers, 11:31) describing an acute devastating illness in Israelites who had eaten quail (that had probably fed on hemlock seeds). Rhabdomyolysis was since then considered uncommon till World War II bombing blitz in London which had subsequently reported in victims of natural catastrophes and individual subjected to crush injury and severe physical exertion (Guis *et al.*, 2005).

1.2 Definition of Rhabdomyolysis

Rhabdomyolysis is a dissolution of skeletal muscles that produces a nonspecific clinical syndrome that results in the extravasation and release of toxic intracellular contents from the myocytes into the circulatory system or plasma (Pesik and Otten, 1996; Criddle, 2003). Skeletal muscles are very resilient and stable in general but can undergo acute destruction and necrosis when pushed beyond their tolerance and ability to self-repair (Criddle, 2003). Muscle fiber necrosis can occur as a primary disorder related to inherited or structural abnormalities of the muscle cells and very often it is secondary to an infection, a toxic agent, an injury or other external cause (Guis *et al.*, 2005). This destruction leads to electrolyte disturbances, hypovolemia, metabolic acidocis, coagulopathies and myoglobinuric renal failure (Criddle, 2003).

1.3 Clinical Presentation of Rhabdomyolysis

In the mildest form, rhabdomyolysis may be limited merely to diffuse muscle aches. However, in its severest form, it presents as myoglobinuria with extensive degree of muscle fiber necrosis and phagocytosis or acute necrotizing myopathy. Myoglobinuria is a sign that often seen after viral infections. The viral infection is self-limited but the myositis may cause permanent deficit. Besides myoglobinuria, fever and sore throat, myalgia and at times swelling in the muscles, generalized muscle weakness or organomegaly may be observed (Dalakas, 2004). When rhabdomyolysis is severe, it may at times be complicated by acute renal failure (Fodili and van Bommel, 2003).

1.4 Diagnosis of Rhabdomyolysis

An elevated serum Creatine Phosphokinase (CPK) is the most sensitive and reliable indicator of muscle injury. Other laboratory diagnosis such as hypocalcemia, hypophosphatemia, hyperuricemia, hyperkalemia and elevated blood urea nitrogen (BUN) and creatinine may be observed as well (Pesik and Otten, 1996).

However, it has been postulated that the strongest and most accurate diagnosis of rhabdomyolysis is still myoglobinuria, as demonstrated by generalized edema, muscle tenderness, weakness and also the brown discolouration of the urine (McDonnell, 2002). Nevertheless, in order to confirm the diagnosis, the urine should be tested for myoglobin (radioimmunoassay is the best technique) and the muscle enzymes should be measured (McDonnell, 2002).

1.5 Etiologies of Rhabdomyolysis

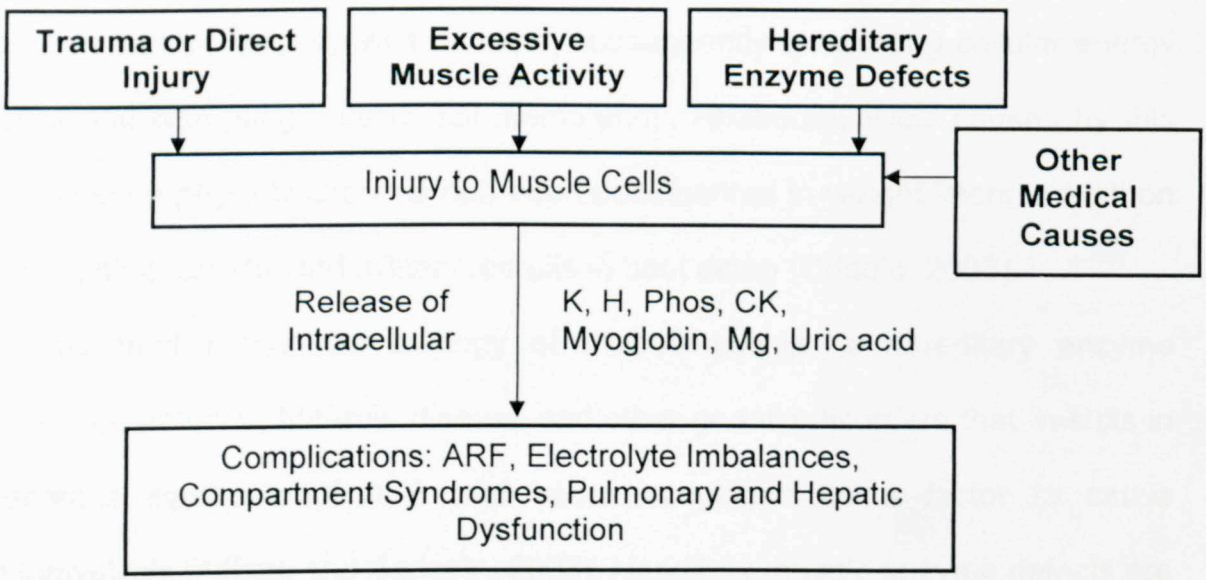

Figure 1: Classification of rhabdomyolysis based on four etiologies (Allison and Bedosole, 2003)

To date, there are multiple and diverse etiologies that are known to be associated with rhabdomyolysis. Allison and Bedsole in 2003 had classified rhabdomyolysis according to etiologies into four broad categories which include trauma or direct muscle injury, excessive muscle activity, hereditary muscle enzyme defects and other medical causes.

Trauma or direct muscle injury can be related to the "crush injury syndrome" which was described in 1941 by Bywater and Beall in victims of World War II bombing raids in London. Dark urine (myoglobinuria), hemoconecentration and acute renal failure were observed to develop subsequently even though the crushed limb were amputated shortly after hospitalization (Allison and Bedsole, 2003; Criddle, 2003; Montgomery *et al.*, 2005).

The second category of the etiologies of rhabdomyolysis is excessive muscle activity. Excessive muscle activity results in a state in which ATP production cannot keep up with demand, subsequently exhausting cellular energy supplies and disrupting muscle cell membranes. Rhabdomyolysis caused by this kind of intense physical exercise had been documented in weight lifters, marathon runners, police cadets, and military recruits in boot camp (Criddle, 2003).

The third recognized etiology of rhabdomyolysis is hereditary enzyme defects. For instance, McArdle disease and other genetic disorders that results in a decrease synthesis of ATP had been recognized as a factor to cause rhabdomyolysis (Allison and Bedsole, 2003). Heriditary muscle enzyme defects are usually suspected in patients with a positive family history of unusual genetic conditions that stimulate rhabdomyolysis such as disorders of carbohydrate and lipid metabolism and a decrease or absence in specific enzymes by histochemical staining (Allison and Bedsole, 2003; Criddle, 2003).

Other medical causes is another etiology of rhabdomyolysis which include drugs and toxins, muscle hypoxia, metabolic and endocrine disorders, viral and bacterial infections, temperature alterations and miscellaneous rare causes (Allison and Bedsole, 2003).

Drugs and toxins is the largest category of etiology under other medical causes (Allison and Bedsole, 2003). Ethanol is known to be the foremost rhabdomyolysis-inducing agent. Besides, use of recreational drugs and stimulants such as heroin, cocaine, sniffing glue and etc, toxins from plants and animals such as snake venoms, toxic mushrooms, ingestion of hemlock and etc, use of pharmaceutical agents such as benzodiazepines, corticosteroids,

immunosuppressants and etc are also documented to be the causes that contributed to rhabdomyolysis (Criddle, 2003).

Another medical cause that may lead to rhabdomyolysis is muscle hypoxia or muscle ischemia, which is the result of prolonged loss of consciousness, compression, immobilization, compartment syndrome, reperfusion injury and arterial or venous occlusions (Allison and Bedsole, 2003; Criddle, 2003).

Metobolic and endocrine disorders is another known etiology of rhabdomyolysis in which hypokalemia and hypophosphatemia had known to be the main contributor to the development of rhabdomyolysis (Allison and Bedsole, 2003).

Other than that, alterated temperature conditions such as malignant hyperthermia, neuroleptic malignant syndrome, and heat stroke, may result in rhabdomyolysis as well (Allison and Bedsole, 2003).

Last but not least, infections also contribute as part of the etiologies of rhabdomyolysis. Cases of rhabdomyolysis had been reported to be associated with various infections such as bacterial infections, viral infections and parasitic infections (Criddle, 2003).

Despite the fact that there are multiple etiologies that had been reported to be associated with rhabdomyolysis, viral infections was the sole etiology that was being focused and investigated in this study.

1.6 Definition of Inflammatory Myopathy

Inflammatory myopathy is a form of muscle weakness which includes polymyositis, dermatomyositis and inclusion body myositis (IBM). In all age groups, dermatomyositis is the most common while polymyositis is the least common (Dalakas and Hohlfeld, 2003) while IBM is the commonest form of inflammatory muscle disease after the age of 50, with a 3:1 male predominance (Mikol and Engel, 2004). IBM can be divided into sporadic type, with a peak incidence after 50 years old, and the familial type, in which rare cases are reported in childhood (Carpenter and Karpati, 2001). Polymyositis is best defined as a subacute myopathy that evolves over weeks to months, affects adults but rarely children (Dalakas and Hohlfeld, 2003). Of these three types of inflammatory myopathies, only IBM and Polymyositis cases will be reviewed in this study.

1.7 Clinical Presentation of Inflammatory Myopathy

IBM can be hereditary or sporadic. In sporadic IBM, it is often characterized by slow progressive which is usually painless and weakness. Most patients remain ambulatory even after 12 years or more while some have serious disability after one to two years. Proximal weakness usually predominates over distal, but the reverse can occur as well. Swallowing, and even facial muscles, may be involved. Sometimes, patients with IBM may be accompanied by autoimmune disease such as Sjogren's syndrome (Carpenter and Karpati, 2001). Besides, atrophy of the deltoids and quadriceps is often present, and weakness of forearm muscles (especially finger flexors) and ankle dorsiflexors are typical. Peripheral neuropathy with loss of deep tendon reflexes may be present in some patients (Rendt, 2001).

Polymyositis is often present with muscle weakness that develops slowly over weeks to months, or at times acutely in rare cases. Patients present with Polymyositis often report difficulty in performing everyday tasks as simple as rising from a chair. In advanced and rare acute cases, dysphagia with choking episodes and respiratory muscle weakness may occur (Dalakas and Hohlfeld, 2003).

1.8 Diagnosis of Inflammatory Myopathy

Routine laboratory studies are one of the methods to diagnose IBM. The erythrocyte sedimentation rate is normal or moderately increased. The serum creatinie kinase level is normal or increased less than ten fold above the upper limit of normal. At times, tests for autoantibodies are positive in 20 to 40 percent of cases (Mikol and Engel, 2004).

Another key criterion in the diagnosis of inflammatory myopathy is by electromyography (EMG). In addition to EMG, another distinct and reliable diagnostic method would be magnetic resonance imaging studies (MRI) (Mikol and Engel, 2004).

Muscle biopsy is another crucial diagnosis of inflammatory myopathy (Dalakas and Hohlfeld, 2003; Christopher-Stine and Plotz, 2004). The biopsies should be processed for frozen sections and with enzyme histochemistry and immunohistochemistry (Dalakas and Hohlfeld, 2003).

1.9 Etiologies of Inflammatory Myopathy

In a literature review by Karen Rendt, a few etiologies had been found to be associated with inflammatory myopathies. These etiologies that based on differential diagnosis include drug or toxin, infection such as bacterial, treponemal, mycobacterial, viral, fungal and parasitic, metabolic myopathy, endocrinopathy, polymyalgia rheumatica, fibromyalgia, sarcoidosis and neuromuscular disorders (Rendt, 2001).

Again, only the etiology of viral infections was inspected in this study.

1.10 Viruses Known to be Associated with Rhabdomyolysis

1.10.1 Association of Dengue Virus (Family *Flaviviridae*) with Rhabdomyolysis

Dengue viruses are divided into four distinct serotypes (1 to 4) and are members of family *Flaviviridae* and genus flavivirus. They are arthropod-borne viruses and are transmitted between humans principally by the mosquito vector, *Aedes aegypti*. Infection can give rise to a wide spectrum of disease manifestations ranging from a mild, self-limiting febrile illness to more severe vascular and haemostatic abnormalities known as haemorrhagic fever-dengue shock syndrome (Seah *et al.*, 1995).

Documented rhabdomyolysis following dengue virus infection had been reported by physicians in Singapore (Lim and Goh, 2005) and Australia (Davis and Bourke, 2004).

A patient presented with a recent dengue virus infection had subsequently developed myalgia and the urine colour had also turned dark red for the following five days. Dipstick analysis was strongly positive for blood (Lim and Goh, 2005).

Davis and Bourke in 2004 had also reported a case which potrayed almost the same symptoms as above. Patient had complained of nausea and retro orbital pain. There was a confluent erythromatous macular rash on his trunk. Besides, CK levels were found to be markedly elevated. Following all these symptoms which strongly represents of that rhabdomyolysis, reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of dengue virus RNA was performed on serum samples of the patient on day 1 of hospitalization and was found to be positive for dengue virus type 2 RNA (Davis and Bourke, 2004).

According to Lim and Goh in 2005, similar cases of rhabdomyolysis which develop following dengue virus infection had previously been reported three times.

1.10.2 Association of West Nile Virus (Family *Flaviviridae*) with Rhabdomyolysis

Patients who developed rhabdomyolysis following West Nile encephalitis and meningitis had also been reported (Montgomery *et al.*, 2005).

Patients with West Nile virus (WNV) neuroinvasive disease (less than 1% of WNV-infected persons) commonly present with muscle weakness that is often assumed to be of neurologic origin. Patients with WNV infection had documented elevated CK levels and generalized muscle weakness. However, the mechanisms behind the elevated CK levels were unclear (Montgomery *et al.*, 2005).

Currently, direct evidence of WNV infection causing myositis has not been documented. As rhabdomyolysis may be induced by multiple etiologies, patients suffered from rhabodmyolysis may be secondary to other factors such as crush tissue injury and muscle tissue hypoxia (Montgomery *et al.*, 2005).

1.10.3 Association of Enteroviruses (Family *Piconaviridae*) with Rhabdomyolysis

Enteroviruses is a large group of virus consist of Coxsackieviruses (group A and B), polioviruses and enteric cytopathogenic human orphan (EHCO) viruses. They are a genus of *piconaviridae* and are wide spread throughout the world (Fodili and van Bommel, 2003).

Severe rhabdomyolysis, ARF, immune-complex mediated acute glomeruloephritis and inflammatory myopathies such as polymyositis and dermatomyositis following Coxsackie viral infection had been reported several times for the past 30 years (Bayatpour *et al.*, 1973; Dunnet *et al.*, 1981; Bowles *et al.*, 1987; Konrad *et al.*, 1993; Beressi *et al.*, 1994; Fodili and van Bommel, 2003).

In most of the cases, similar clinical signs and symptoms had been reported. For instance in a case report documented by Fodili and van Bommel in 2003, severe muscular pain, discoloured coca cola-like urine had been developed in a patient with five days history of malaise, sore throat, fever up to 40^0C and headache. Viral serological tests were performed which strongly showed a 4 fold rise in Coxsackie B_2 antibody while no significant rise in other viral antibody. This had strongly suggested a recent Coxsackie virus infection had occurred on that patient (Fodili and van Bommel, 2003). Besides, severe myalgia with swelling and

tenderness of the limb muscle (Dunnet *et al.*, 1981), sore throat, diffuse muscle pain and weakness, enlarged tonsils, cervical lymphadenopathy, myoglobinuria, elevated serum CK level (Konrad *et al.*, 1993) had also been reported.

Coxsackie A_9, A_{16}, and B_{2-6} were among the serotypes associated with severe rhabdomyolysis, acute renal failure and acute glomeruloephritis that had been reported (Bayatpour *et al.*, 1973; Dunnet *et al.*, 1981; Konrad *et al.*, 1993; Fodili and van Bommel, 2003).

Besides Coxsackieviruses group A and B, acute rhabdomyolysis in a well-trained long distance runner associated with a large rise in echovirus 9 titers during a two-week period had also been reported (Josselson *et al.*, 1980).

A muscle biopsy on the day after admission to hospital had demonstrated diffuse necrosis, with loss of striation and cytoplasmic vacuolation. Blood vessels too, contained polymorphonuclear leucocytes. Electron microscopy study confirmed the diagnosis of acute rhabdomyolysis. The greater than fourfold rise in echovirus 9 titers in the presence of febrile illness which was unassociated with bacterial infection and in the absence of elevations of other viral titers was strong evidence of echovirus infection at the onset of the patient's illness (Josselson *et al.*, 1980).

1.10.4 Association of Influenza Virus (Family *Orthomyxoviridae*) with Rhabdomyolysis

Influenza viruses consist of three types which are A, B and C. Most pandemics of influenza are associated with type A though at times type B may also prevalent along with type A in the influenza-related ourbreaks. Type C generally

does not cause severe outbreaks and it only causes mild illness. The typing of influenza viruses is based on antigenic differences on the nuclear and matrix proteins of the viruses. For example, influenza type A is subtyped on the basis of antigenic differences on the external glycoproteins, the hemagglutinin (H) and the neuraminidase (N) proteins (Poddar, 2002).

Rhabdomyolysis following infection with influenza B virus had been reported in year 1993 which involved a young boy who had presented with severe rhabdomyolysis and bilateral compartment syndrome due to infection with influenza B virus (Paletta *et al.*, 1993). Besides, sporadic cases of myoglobinuria and acute renal failure during the course of influenza virus infection had also earlier been reported in 1979 (Cunningham *et al.*, 1979). In 1976, Shenouda and Hatch had reported four cases of acute renal failure following influenza A viral infection during a recent mid-winter epidemic (Shenouda and Hatch, 1976).

Viral rhabdomyolysis is an acute condition that normally presents as a viral syndrome with severe myalgias (Paletta *et al.*, 1993). According to the specific case reports above which had documented the association of Influenza viruses (A and B) with rhabdomyolysis, patients usually develop complications such as dark-coloured urine (Paletta *et al.*, 1993), generalized muscle aching and weakness, myoglobinuria and typical flu-like illness to oliguric renal failure (Cunningham *et al.*, 1979). In addition, other muscle components including creatine, serum glutamic oxaloacetic transaminase (SGOT), lactic dehydrogenase (LDH), CPK, potassium and many others are also released into the circulation (Shenouda and Hatch, 1976).

For all the cases reported above, most have shown elevated and significant increase in titers for influenza A virus which suggest a strong association between influenza A infection and the complications that preceded.

1.10.5 Association of Herpesviruses (Family *Herpesviridae*) with Rhabdomyolysis

Herpesviruses are enveloped double stranded DNA viruses of the family Herpesviridae and they are found throughout the animal kingdom, and nearly 100 species have been isolated and partially characterized (VanDevanter *et al.*, 1996). Thus far, there are 9 herpesviruses that have been isolated from humans. These herpesviruses are herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), cytomegalovirus (CMV), Varicella zoster virus (VZV), Epstein-Barr virus (EBV), and Human herpesviruses 6A, 6B, 7 and 8 (HHV-6A, HHV-6B, HHV-7 and HHV-8 (Roizman and Pellett, 2001).

Though cases of rhabdomyolysis following herpses virus infection is not reported as frequent as other virus families, there had been few case reports in the 1970s and 1980s which documented the association of herpes virus infection and rhabdomyolysis.

In 1974, Schlesinger et al. reported two cases of myoglobinuria associated with herpes simplex (HSV) and Epstein-Barr (EBV) viruses, respectively. Significant complications include residual muscle atrophy, acute renal failure, pharyngitis and progressive darkening of the urine (Schlesinger *et al.*, 1978).

In addition, a case of rhabdomyolysis, myositis, and myoglobinuria associated with cytomegalovirus (CMV) (Hughes and Hunt, 1984) and another

case of EBV infection coupled with rhabdomyolysis, myoglobinuria and nonoliguric renal failure were also reported (Friedman and Libby, 1986) in the mid 1980s.

The pathogenesis of the rhabdomyolysis during an acute viral illness had not been disvovered and no morphologic evidence of direct viral involvement could be found in these cases (Schlesinger *et al.*, 1978; Friedman and Libby, 1986). Though tubular aggregation particles had been found in herpes simplex infection, but they are thought to represent a cellular response rather than actual viral particles (Schlesinger *et al.*, 1978).

1.10.6 Association of Human Immunodeficiency Virus (HIV) with Rhabdomyolysis

For the past ten over years (1994-2006), there had been numerous cases reported whereby patient with primary HIV infection were presented with acute rhabdomyolysis (Chariot *et al.*, 1994; Rastegar *et al.*, 2001; McDonagh and Holman, 2003).

Despite there were many studies that showed the correlation between acute rhabdomyolysis and HIV infection, HIV had not been investigated in this study as there were no HIV patient histories reported among the cases of local patients presented with acute rhabdomyolysis.

1.11 Association of Viral Infection with IBM

To date, there has not been a single case reported on IBM to have a substantial association with viral infection. As IBM is a milder form of myopathy compared to rhabdomyolysis, the association of it with viral infection had not yet been well documented.

The attempt to investigate the association between IBM and Paramyxoviruses (mumps virus) on the muscle biopsies of patients with IBM by using immunological techniques such as immunocytochemistry and ISH revealed no association (Nishino *et al.*, 1989; Kallajoki *et al.*, 1991). This had suggested that either mumps virus is not the etiologic agent in IBM (Kallajoki *et al.*, 1991) or the techniques used were not sufficiently sensitive to detect it (Nishino *et al.*, 1989).

1.12 Association of Viral Infection with Polymyositis

Though several viruses (coxsackieviruses, influenza, parvoviruses, paramyxoviruses, cytomegaloviruses, Epstein-Barr virus) have been reported to be indirectly associated with myositis, PCR had not been able to amplify viral genome from the muscle tissues of these patients (Dalakas and Hohlfeld, 2003).

However, in another report, CMV and EBV had been documented to possess an association in patients with interstitial lung disease in Polymyositis (Hashimoto et al., 1995).

1.13 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

As most of the viruses known to be associated with rhabdomyolysis and IBM are RNA viruses, RT-PCR had been employed to be the predominant method to be used in this study to detect the presence of a few specific types of viral genome, if any.

RT-PCR is a method used to amplify cDNA copies of RNA. It is sensitive and versatile and is often used to retrieve and clone 5' and 3' termini of mRNAs and to generate large cDNA libraries from very small amount of mRNA which is often obtained from limited tissue samples (Sambrook and Russell, 2001). The RT step can be primed by using specific primers, such as gene specific primers, random hexamers or oligo dT primers (Bustin, 2000).

First Strand Synthesis:

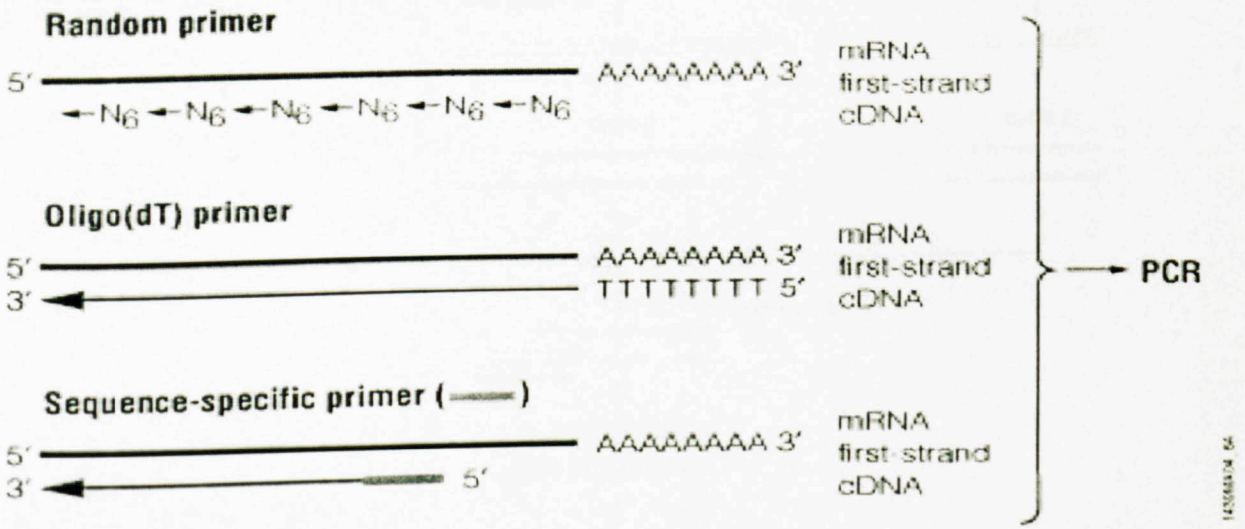

Figure 2: Three different RT steps

(Adapted from: www.promega.com/paguide/chap1.htm)

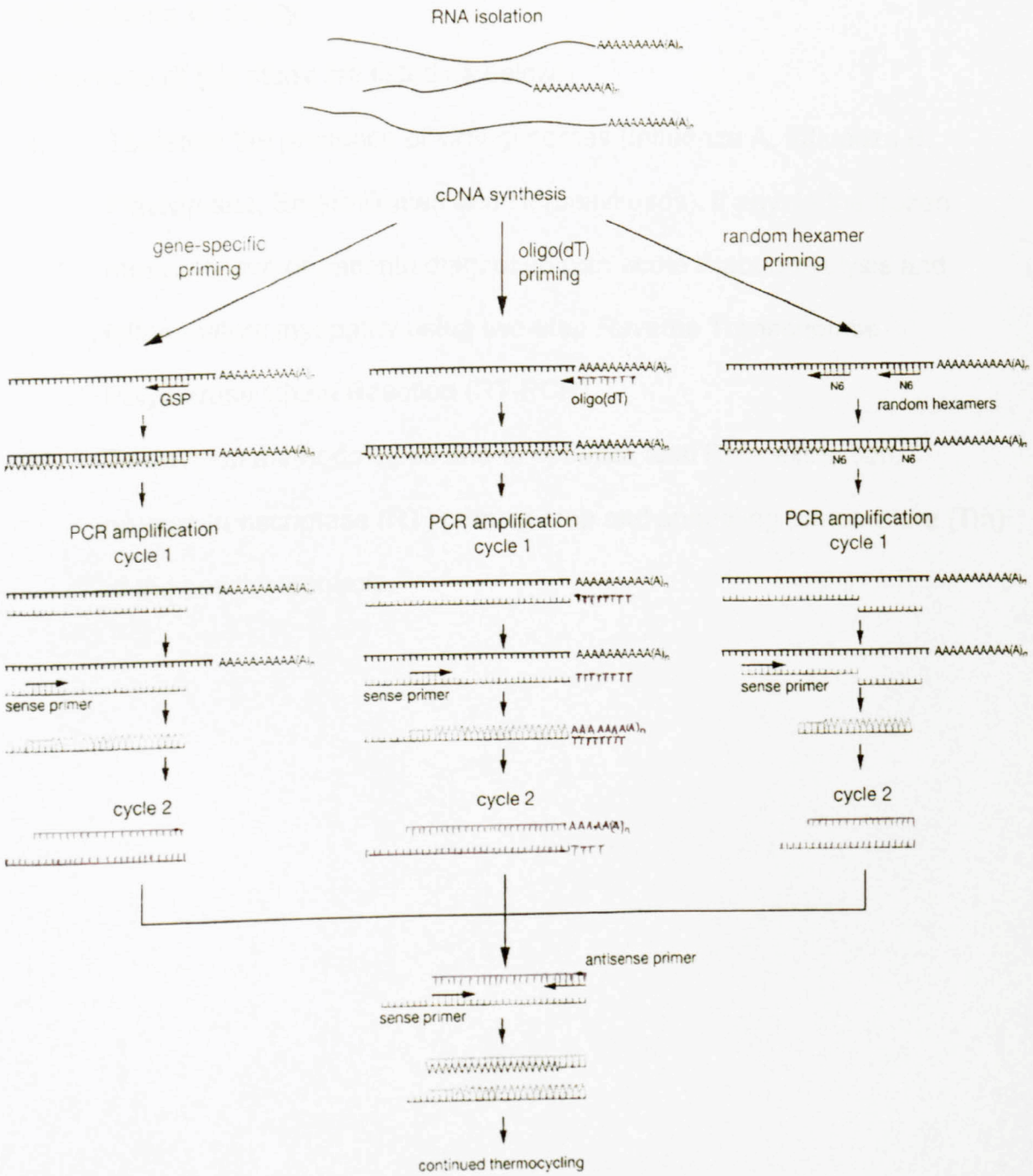

Figure 3: Various methods for the amplification of RNA by RT-PCR (Sambrook and Russell, 2001)

1.14 Objectives of Study

The objectives of this study are listed as below:-

(i) To detect the presence of viral genomes (Influenza A, Influenza B, Flaviviruses, Enteroviruses and Herpesviruses), if any, in the frozen muscle tissue of patients diagnosed with acute rhabdomyolysis and inflammatory myopathy using two-step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

(ii) To develop methodologies and to optimize total RNA extraction, reverse transcriptase (RT) priming step and annealing temperature (Tm) of the positive controls.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Clinical Samples and Data Collection

A total of 13 cases of previously diagnosed acute rhabdomyolysis and inflammatory myopathy with inclusion body myositis and polymyositis based on the histological findings (histology sections were viewed by Prof. KT Wong), were retrieved from the anatomic pathology reports files in the Department of Pathology, University of Malaya Medical Centre (UMMC), Malaysia. Among the 13 cases, there were 5 cases of acute rhabdomyolysis and 8 cases of inflammatory myopathy from years 1994 to 2006.

Frozen muscle biopsies embedded with OCT (Optimal Cutting temperature) compound were obtained from the Department of Pathology, UMMC, Malaysia. The patients' samples were transferred quickly from -80°C freezer to -20°C cryostat. This is to prevent ice crystal formation and damage of the frozen muscle biopsy when exposed too long to room temperature. The cryostat was decontaminated before and after used for each tissue block by swapping the cryostat with 70% ethanol. Each sample was sectioned 8 microns for each section and about 9 to 10 sections were picked and stored in two sterile 1.5 ml microcentrifuge tubes for subsequent DNA and RNA extraction.

Besides that, tissue sections for all the clinical samples were also collected for immunohistochemistry (IHC) stained.

2.2 Preparation of Positive Controls

2.2.1 Extraction of Viral RNA

Influenza A and B infected MDCK cell cultures supernatant were obtained from the National Public Health Laboratory, Sungai Buloh, Selangor (a gift by Dr. Chua Kaw Bing). Dengue serotype 1,3 and 4 MDCK cell cultures supernatant were stored in the lab while Dengue serotype 2 MDCK cell culture supernatant was obtained from Mr. Hj. Osmali Osman from the Department of Microbiology. They were extracted by using Viral RNA extraction kit (Search Corp., California, U.S.). This viral extraction kit is designed for the isolation of viral RNA from less than 200µl biological liquid or cells in suspension at a concentration of less than or equivalent to $1X10^5$ cells/ml.

Viral genomic RNA was extracted from the culture fluid of infected cells by using this Viral RNA extraction kit (Search Corp., California, U.S.) according to the manufacturer's procedures. Briefly, 200µl of culture fluid was pipetted into a 1.5 ml microcentrifuge tube and three volumes (600 µl) of ZR Viral RNA buffer was then added to the tube. The mixture was then transferred to a Zymo-Spin IC™ Column in a collection tube and centrifuged at 10,000 rpm for 1 minute. The flow-through was then discarded and the column was washed with 300 µl of RNA wash buffer and centrifuged at 10,000rpm for 1 minute. The flow-through was discarded. This step was repeated again. After that, Zymo-Spin IC™ Column was placed into a provided RNase-free microcentrifuge tube. About 6-10µl of the provided DNase/RNase-Free water was added to the column and let it stand in the room temperature for 1 minute. Then, it was centrifuged for 1 minute to elute out the RNA. RNA was stored at -80°C before used.

2.2.2 Extraction of Viral DNA

Virus stock for Cytomegalovirus was obtained from Mdm. Poh Sim Hooi (Department of Microbiology) while Epstein-Barr virus stock was obtained from Mr. Andrew Lee Lin Kiat (Department of Pathology). The viral DNA was extracted using QIAamp ® DNA Mini Kit (Qiagen, Germany) according to the manufacturer's procedures. Briefly, 20 µl of QIAGEN Protease (or Proteinase K) was pipetted into the bottom of a 1.5 ml microcentrifuge tube and 200 µl of sample (culture of Cytomegalovirus and Epstein-Barr virus) was then added to the microcentrifuge tube. This was followed by adding 200 µl of Buffer AL to the sample and the mixture was mixed by pulse vortexing for 15 seconds. The mixtures were incubated at 56^0C for 10 minutes. The microcentrifuge tube was then briefly centrifuged to remove drops from the inside of the lid. 200µl of 100% ethanol was added to the sample, and mixed again by pulse-vortexing for 15 seconds. After mixing, it was briefly centrifuged to remove drops from the inside of the lid. The mixture was then carefully applied to the QIAamp spin column (in a 2ml collection tube) without wetting the rim. The cap was then closed and centrifuged at 8000 rpm for 1 minute. The QIAamp spin column was then placed in a clean 2 ml collection tube. The spin column was then carefully opened and 500µl of Buffer AW1 was added without wetting the rim. The cap was closed and centrifuged at 8000 rpm for 1 minute. The spin column was placed in a clean 2ml collection tube provided and the collection tube containing the filtrate was discarded. After that, the spin column was carefully opened and 500 µl of Buffer AW2 was added without wetting the rim. The cap was closed and centrifuged at full speed (14,000 rpm) for 3 minutes. Lastly, the spin column was placed in a new 2 ml collection tube. The

spin column was carefully opened and 200 µl of Buffer AE was added. It was then incubated at room temperature for 1 minute, before centrifuging it at 8000 rpm for 1 minute.

2.3 Comparison of Two Total RNA Extraction Methods

One of the methods was performed by using TRIzol ® Reagent (total RNA extraction kit) while the other method was performed by combining Proteinase K (Promega, Madison, USA) digestion followed by TRIzol ® Reagent. These two methods differed only by their initial few steps while the subsequent steps were the same. The results obtained in terms of total RNA yield and purity of the RNA (A260/A280) were analyzed and the method which yielded higher RNA value and purer RNA would be selected as the preferable RNA extraction method to be used for the 13 clinical samples.

2.3.1 Extraction of Total RNA Using TRIzol ® reagent

The EV71 infected mice limb skeletal muscles were previously stored in -80°C. Approximately 0.02 g of muscle tissues were used in this experiment. Before digestion, muscle tissues were mechanically minced into small pieces using disposable surgical blade with a holder on a Petri dish. The muscle tissues were then transferred into a 1.5 ml tube which contained 1 ml of TRIzol ® reagent and incubated for 15 min at RT to allow full muscle digestion. Then, 0.2 ml of chloroform was added and the tube was shaken vigorously for 15 seconds and incubated at room temperature (RT) for 15 minutes. After this, the samples were centrifuged at 10,000 rpm at 4°C for 15 minutes. This was to allow complete and

thorough separation of the 3 phases in the tube: aqueous phase on top, interphase in the middle and protein at the bottom.

The aqueous phase which contained RNA was transferred to a new 1.5 ml tube and isopropanol was precipitated (0.5 ml of isopropanol per of 1 ml TRIzol ® reagent) for 10 minutes at RT. Tube was then centrifuged at 10,000 rpm at 4°C for 10 min. A gel like white pellet which contained RNA was seen in the bottom of the tube. The supernatant was removed and RNA pellet was washed with 75% ethanol (twice, 5 minutes each) and centrifuged at 10,000 rpm at 4°C for 5 minutes. The RNA pellet was dried in a fume hood for about 1 hour before it was dissolved in 20 µl of RNase free water (Double distilled Mili Q water).

2.3.2 Extraction of Total RNA using Proteinase K and TRIzol ® Reagent

The EV 71 infected mice limb skeletal muscles were initially stored in the -80°C freezer. The OCT compound was removed from the muscle tissues by adding in 70% ethanol. Following this step, muscle tissues were then transferred into a 1.5 ml tube which contained tissues lysis buffer (10 mM Tris pH 8, 100mM EDTA, 100mM NaCl, 0.5% SDS) and Proteinase K was added to a final concentration of 0.1mg/ml. The new tube was then incubated in the water bath at 55°C for 3 hours to allow complete digestion of the muscle tissue. TRIzol ® reagent was added after 3 hours of incubation. The tube was taken out and let it cool to RT and 800 µl of TRIzol ® Reagent was added into the tube. After that, it was incubated in room temperature for 15 minutes to allow the full muscle tissue digestion. Subsequent procedures were same as above (section 2.3.1).

thorough separation of the 3 phases in the tube: aqueous phase on top, interphase in the middle and protein at the bottom.

The aqueous phase which contained RNA was transferred to a new 1.5 ml tube and isopropanol was precipitated (0.5 ml of isopropanol per of 1 ml TRIzol ® reagent) for 10 minutes at RT. Tube was then centrifuged at 10,000 rpm at 4°C for 10 min. A gel like white pellet which contained RNA was seen in the bottom of the tube. The supernatant was removed and RNA pellet was washed with 75% ethanol (twice, 5 minutes each) and centrifuged at 10,000 rpm at 4°C for 5 minutes. The RNA pellet was dried in a fume hood for about 1 hour before it was dissolved in 20 µl of RNase free water (Double distilled Mili Q water).

2.3.2 Extraction of Total RNA using Proteinase K and TRIzol ® Reagent

The EV 71 infected mice limb skeletal muscles were initially stored in the -80°C freezer. The OCT compound was removed from the muscle tissues by adding in 70% ethanol. Following this step, muscle tissues were then transferred into a 1.5 ml tube which contained tissues lysis buffer (10 mM Tris pH 8, 100mM EDTA, 100mM NaCl, 0.5% SDS) and Proteinase K was added to a final concentration of 0.1mg/ml. The new tube was then incubated in the water bath at 55°C for 3 hours to allow complete digestion of the muscle tissue. TRIzol ® reagent was added after 3 hours of incubation. The tube was taken out and let it cool to RT and 800 µl of TRIzol ® Reagent was added into the tube. After that, it was incubated in room temperature for 15 minutes to allow the full muscle tissue digestion. Subsequent procedures were same as above (section 2.3.1).

2.4 Extraction of Total DNA Using Proteinase K and Phenol-Chloroform

DNA was extracted using proteinase K and phenol-chloroform method. Briefly, before digestion, muscle tissues were mechanically minced into small pieces using disposable surgical blade with a holder on a Petri dish. The muscle tissues were then transferred into a 1.5 ml tube which contained tissues lysis buffer (10 mM Tris pH 8, 100mM EDTA, 100mM NaCl, 0.5% SDS) and Proteinase K was added to a final concentration of 0.1 mg/ml. Homogenates were incubated in a water bath for 3 hours at 55°C to permit complete tissues digestion. Lysate was cool to RT prior to phenol-chloroform purification. An equal volume of phenol-chloroform was added and tube was shaken vigorously for about 15 seconds before centrifuged at 13,000 rpm for 10 minutes.

Aqueous phase of each tube which contained DNA was transfer into a new 1.5 ml tube and the extraction was repeated for one more time. After the second extraction with phenol-chloroform, DNA was precipitated with 0.1 volume of sodium acetate (3 M, pH 5.2) and 2 volumes of absolute ethanol at -20°C for overnight.

After overnight precipitation, tube containing precipitated DNA was centrifuged at 13,000 rpm for 20 minutes at RT. The supernatant was removed and DNA pellet was washed with 70% ethanol twice and centrifuged at 13,000 rpm at RT for 5 minutes (after each washed). DNA pellet was dried in a fume hood for about 1 hour before it was dissolved in 20 µl of DNase-RNase free water (Double distilled Milli Q water).

2.5 Reverse Transcriptase (RT) Reaction- Comparison of Three Different Priming Steps Using Oligo dT Primers, Random Hexamer and Gene Specific Primers

The RT reaction was carried out to convert RNA to cDNA. Generally there are three types of primers used for the priming step in RT, which are oligo dT primers, random hexamer and gene specific primers. In this study, the sensitivity and specificity of these three primers were compared.

The viral genome was reverse transcribed using MMLV reverse transcriptase under the following conditions: RNA (1 µg), 4 µl 1X Reaction Buffer, 0.5 mM dNTPs, 2 U RNase Inhibitor (Promega, Madison, USA), 0.5 U MMLV reverse transcriptase (Ambion, UK), double distilled Milli Q water, 5.0 µM of oligo dT primers (Ambion, UK); 2.5µM of gene specific primers or 50 µM of random hexamer (Promega, Madison, USA) were respectively added to a final volume of 20 µl and heated at 42°C for 1 hour using Perkin Elmer 480 thermal cycler (USA).

Table 1: Reagents for RT Reactions

NO	Reagent	Source	Volume(µl)	Stock [ ]	Working [ ]	Final [ ]
1	5X reaction buffer	Promega, Madison, USA	4.0	-	-	1X
2	dNTP mix	Promega, Madison, USA	1.0	10mM	10mM	0.5mM
3	MMLV Reverse Transcriptase	Ambion, UK	1.0	10 Unit/µl	10 Unit/µl	0.5 Unit/µl
4	Rnase inhibitor	Promega, Madison, USA	1.0	40 Unit/µl	40 Unit/µl	2 Unit/µl
5	Oligo dT	Ambion, UK	2.0	50µM	50µM	5µM
	Random hexamer	Promega, Madison,USA	2.0	500µM	500µM	50µM
	Gene specific primer	Proligo, Singapore	2.0	25µM	25µM	2.5µM
6	Double distilled Mili-Q Water	In house prepared		-	-	-
7	RNA template	Extracted from muscle biopsies	-	-	-	1µg
	Total:		**20.0**			

2.6 Comparison of Two Different PCR buffers and Different Proportions of Primer Concentrations in Duplex PCR

Two different PCR buffers were compared using the EV71 viral RNA as template. These two buffers were Taq buffer with $(NH_2)SO_4$ (Fermentas, Hanover, MD, USA) and KCl buffer (Fermentas, Hanover, MD, USA). These two buffers were compared in terms of their consistency and sensitivity during each PCR. Different proportions of EV71 and β actin primer concentrations were investigated in duplex PCR in order to determine the best combination of these two pairs of primers to produce the most optimum result.

2.7 Internal control

In this study, β actin which is known as housekeeping gene was used as an internal control. β actin genes were amplified by using β actin primers under the following condition: 1X KCl buffer (Fermentas, Hanover, MD, USA), 3mM $MgCl_2$ (Fermentas, Hanover, MD, USA), 0.2mM dNTP mix (Promega, Madison, USA), 0.05U Taq Polymerase (Fermentas, Hanover, MD, USA), 0.5μM β actin forward primer (Proligo, Singapore), 0.5μM β actin reverse primer (Proligo, Singapore), in house prepared double distilled Mili-Q Water and 2μl of cDNA template were respectively added to a final volume of 25μl and subjected to PCR according to the condition as shown in Table 2.

Table 2: Cycling condition for internal control using β actin genes

1. Initial denaturation	94^0C for 5 minutes
2. Denaturation	94^0C for 50 seconds
3. Annealing	55^0C for 1 minute
4. Extension	72^0C for 1 minute
5. Final extension	72^0C for 10 minutes

Note: Step 2 to 4 were repeated for 34 cycles before proceeding to step 5

2.8 Polymerase Chain Reaction (PCR) and Optimizartion of Positive Controls' Annealing Temperature

PCR was carried out in 25 µl reactions containing 1X KCl buffer (Fermentas, Hanover, MD, USA), 3mM $MgCl_2$ (Fermentas, Hanover, MD, USA), 0.2mM dNTP mix (Promega, Madison, USA), 0.05Unit/µl Taq Polymerase (Fermentas, Hanover, MD, USA), 0.5µM forward primer (Proligo, Singapore), 0.5 µM reverse primer (Proligo, Singapore), in house prepared double distilled Mili-Q Water and 2µl of cDNA template.

The reaction mixtures were prepared as master mix on ice before aliquoting them into thin-walled PCR tubes. DNA amplification was performed using Mastercycler Gradient thermal cycler (Eppendorf AG, Hamburg, Germany). PCR was subjected to denaturation at 95 °C for 5 minutes followed by 35 cycles of denaturation at 95 °C, annealing temperature according to the optimization of respective virus primers and extension at 72 °C for 1 minute each. A final

extension for 5 minutes was performed after the last cycle. Amplified products were analyzed by electrophoresis on 1.5 % agarose gels containing 0.5 µg/ml ethidium bromide.

In this study, all the positive controls were optimized at four annealing temperatures (55, 60, 65 and 68^{0}C) to determine the optimum annealing temperature. The PCR conditions were the same as mentioned earlier in this section and the detailed cycling conditions were shown in Table 3. The most optimum annealing temperature for each type of virus was determined according to the gel electrophoresis result which produced the most intense band.

Table 3: Cycling conditions for DNA and RNA viruses using five different pairs of primers

	EV	Inf A	Inf B	Flavi	Herpes
1. Initial Denaturation	94^0C for 5 minutes	94^0C for 5 minutes	94^0C for 5 minutes	94^0C for 5 minutes	94^0C for 5 minutes
2. Denaturation	94^0C for 50 seconds	94^0C for 50 seconds	94^0C for 50 seconds	94^0C for 50 seconds	94^0C for 30 seconds
3. Annealing	Optimized	Optimized	Optimized	Optimized	Optimized
4. Extension	72^0C for 1 minute	72^0C for 1 minute	72^0C for 1 minute	72^0C for 1 minute	72^0C for 1 minute
5. Final extension	72^0C for 10 minutes	72^0C for 10 minutes	72^0C for 10 minutes	72^0C for 10 minutes	72^0C for 7 minutes

Note: Steps 2 to 4 were repeated for 34 cycles before proceeding to step 5. Nested PCR (1^0 and 2^0 PCR) was performed for Herpesviruses.

Abbreviation: **EV**, Enterovirus

Inf A, Influenza A

Inf B, Influenza B

Flavi, Flaviviruses

Herpes, Herpesviruses

Table 4: Reaction mixtures for PCR

NO	Reagent	Source	Volume(µl)	Stock [ ]	Working [ ]	Final [ ]
1	KCl buffer	Fermentas, Hanover, MD, USA	2.5	-	-	1X
2	$MgCl_2$	Fermentas, Hanover, MD, USA	3.0	25mM	25mM	3mM
3	dNTP mix	Promega, Madison, USA	0.5	10mM	10mM	0.2mM
4	Taq Polymerase	Fermentas, Hanover, MD, USA	0.25	5 Unit/µl	5 Unit/µl	0.05 Unit/µl
5	Forward Primer	Proligo, Singapore	0.5	100µM	25µM	0.5µM
6	Reverse Primer	Proligo, Singapore	0.5	100µM	25µM	0.5µM
7	Double distilled Mili-Q Water	In house prepared	15.75	-	-	-
8	cDNA template	From RT reactions	2.0	-	-	-
	Total:		**25.0**			

Table 5: Primer sequence for the amplification of five different species of viruses

Primer	Primer Sequence	Product (bp)	Reference
Enterovirus		500	Arola and Kalimo et al., 1995
Forward	5'-GTA-MCY-TTG-TRC-GCC-WGT-TT-3'		
Reverse	5'-GAA-ACA-CGC-ACA-CCC-AAA-GTA-3'		
Influenza A		311	Elden and Schipper et al., 2001
Forward	5'-CCG-AGA-TCG-CAC-AGA-GAC-TTG-AAG-AT-3'		
Reverse	5'-GGC-AAG-TGC-ACC-AGC-AGA-ATA-ACT-3'		
Influenza B		159	Poddar, 2002
Forward	5'-AAA-TAC-GGT-GGA-TTA-AAT-AAA-AGC-AA-3'		
Reverse	5'-CCA-GCA-ATA-GCT-CCG-AAG-AAA-3'		
Flavivirus		511	Lanciotti and Calisher et al., 1991
Forward	5'-TCA-ATA-TGC-TGA-AAC-GCG-CGA-GAA-ACC-G-3'		
Reverse	5'-TTG-CAC-CAA-CAG-TCA-ATG-TCT-TCA-GGT-TC-3'		
Herpesvirus		700-800	Vandevanter and Warrener et al., 1996
1^0 Forward 1	5'-GAY-TTY-GCN-AGY-YTN-TAY-CC-3'		
1^0 Forward 2	5'-TCC-TGG-ACA-AGC-AGC-ARN-YSG-CNM-TNA-A-3'		
1^0 Reverse	5'-GTC-TTG-CTC-ACC-AGN-TCN-ACN-CCY-TT-3'		
2^0 Forward	5'-TGT-AAC-TCG-GTG-TAY-GGN-TTY-ACN-GGN-GT-3'	200-300	
2^0 Reverse	5'-CAC-AGA-CGT-RTC-NCC-RTA-DAT-3'		

2.9 Agarose Gel Electrophoresis (AGE) and Photography

Soon after PCR, a 1.5% agarose gel was casted. Gel casting was performed by addition of 0.45g of agarose powder (Promega, Madison, USA) to 30ml of TBE (Tris-Borate-EDTA) buffer into a conical flask and the mixture was heated on a hot plate to boiling temperature until all the agarose powder had melted. The mixture was then allowed to cool down to room temperature and 1.5 µl of ethidium bromide (10mg/ml) was added and mixed properly. This mixture was then poured into a gel tank with a 12-slot comb and allowed to set for at least 30 minutes.

Amplified products (DNA) were loaded using the loading dye (Fermentas, Hanover, MD, USA). Loading dye consisted of a mixture of glycerol, bromophenol blue and xylene cyanol dyes. Glycerol helped to weigh down the amplified products so that they are easily sank in the well while Bromophenol Blue and xylene cyanol mixture served as markers to monitor the gel run.

AGE was performed at 90 volts for 45 minutes. A 100bp ladder was electrophoresed together with the amplified products. This was to enable the quantification of amplicons of the amplified products to be made efficiently. After 45 minutes run, the gel was visualized by using UV transilluminator (Herolab, UVT-20M). The results were recorded according to the presence and absence of amplified products and after that Polaroid photographs were taken for permanent recording.

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minutes and then washed twice by using TBS buffer. The sections were then developed using Diaminobenzidine (DAB) (Dako, Glostrup, Denmark) and counter stained with Harris Hematoxylin. Sections were dehydrated and cleared before mounted with DPX mounting media.

CHAPTER 3

RESULTS

RESULTS

3.1 Comparison of Two RNA Extraction Method

RNA extraction using only TRIzol ® Reagent and combination of proteinase K followed by TRIzol ® Reagent using the same amount of EV71-infected mouse limb muscle were compared in this study.

The results were compared in terms of RNA yield and RNA purity (A260/A280). From the results as shown in the table 6, though the RNA purity using both methods did not differ much, however the RNA yield is much higher when using proteinase K followed by TRIzol ® Reagent compared to merely using TRIzol ® Reagent.

Table 6: Concentration and purity of RNA using two different extraction methods

	Concentration (µg/ml)	**Purity (A260/A280)**
EV71 infected limb muscle of mice + TRIzol ® Reagent	443.0	1.74
EV71 infected limb muscle of mice + Proteinase K + TRIzol ® Reagent	1714.5	1.78

3.2 Comparison of Three Different RT Primers (Oligo dT, Random Hexamer and Gene Specific Primers)

RT priming steps were performed by using three types of primers which include Oligo dT, random hexamer and gene specific primers on three separate samples consist of similar amount of EV71 infected limb muscle of the mice. After this, PCR was performed on these three samples.

The gel electrophoresis result showed that equally strong and luminous bands were obtained from RT steps using both gene specific primers and random hexamer. However, RT step using Oligo dT had failed to produce a band as intense as gene specific primers and random hexamer.

Despite producing the same band intensity, random hexamer was chosen as the preferable RT steps to be performed on the clinical samples later on as it has higher sensitivity to prime the RNA. Besides, random hexamer also have the advantage to enable simultaneous amplification of target bands using the same cDNA after RT is being performed.

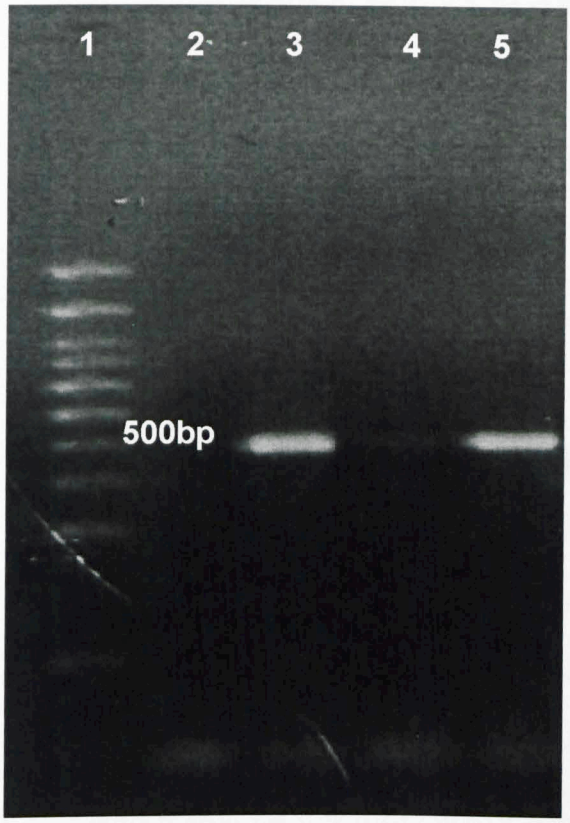


Figure 4: Gel electrophoresis of amplified products using three different RT primers

Lane 1: 100 bp DNA ladder

Lane 2: Negative Control

Lane 3: EV71 amplified products using gene specific primers in RT step

Lane 4: EV71 amplified products using oligo dT primers in RT step

Lane 5: EV71 amplified products using random hexamer in RT step

3.3 Optimization of Duplex PCR Using Different Buffers and Different Proportion of Primer Concentrations

After RT steps whereby cDNA had been obtained, a duplex PCR was performed using both EV degenerate primers and β actin (housekeeping genes) primers. Two types of buffer (Taq buffer with $(NH_2)SO_4$ and KCl buffer) were also tested and compared.

Two sets of primers (EV71 primers and β actin primers) together with one of the two types of buffers were applied at the same time. The effect of different proportion of primer concentrations and also each of the buffers used in the duplex PCR reaction was elucidated based on the intensity of the targeted bands.

It was found that a slight difference on the proportion of primers did not influence the migration of the DNA as well as the intensity of the bands in duplex PCR as both β actin and EV71 targeted region appeared as two significant bands on the same lane in the gel.

However, the buffer used was found to have an effect in influencing the intensity of the bands. KCl buffer was found to possess an ability to generate a slightly stronger and more intense bands compared to Taq buffer with $(NH_2)SO_4$.

Thus, KCl buffer had chosen to be used in the subsequent amplification of the clinical samples during PCR later on.

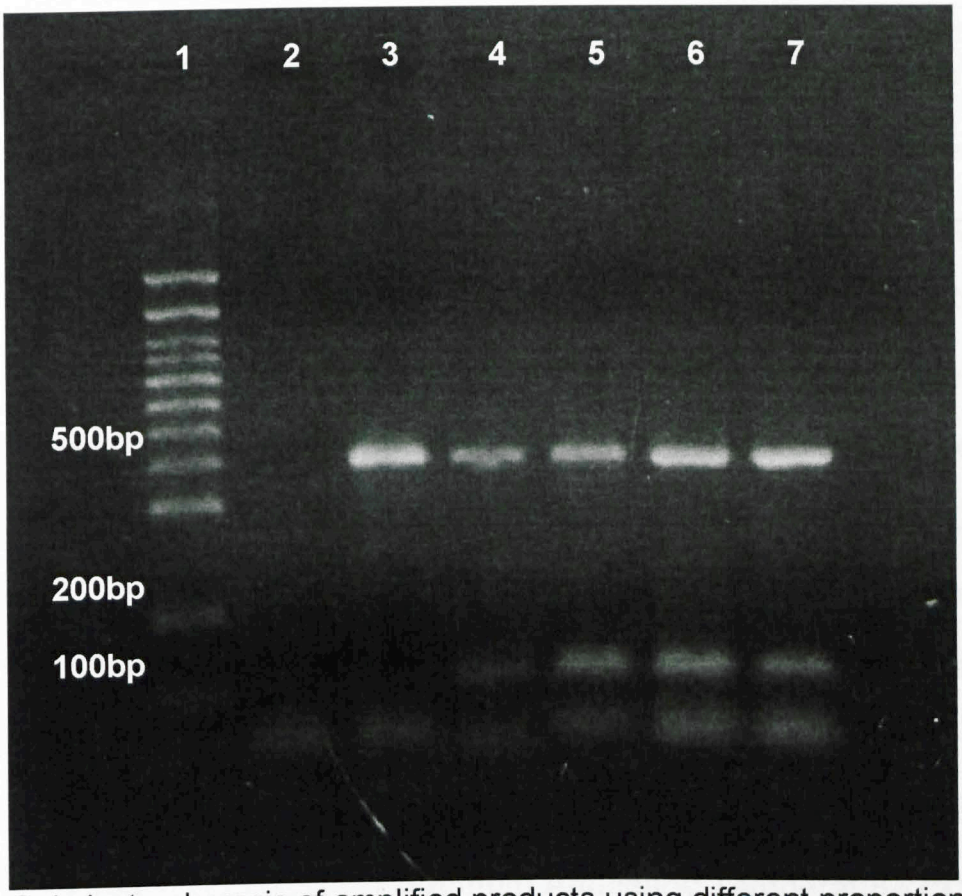


Figure 5: Gel electrophoresis of amplified products using different proportion of primer concentrations and different buffers in duplex PCR

Lane 1: 100bp DNA ladder

Lane 2: Negative control

Lane 3: Amplified products using 0.5μM EV primers only

Lane 4: Amplified products using 1.0μM EV primers & 0.2μM β actin primers } Using Taq buffer with $(NH)_2SO_4$

Lane 5: Amplified products using 0.5μM EV primers & 0.5μM β actin primers

Lane 6: Amplified products using 0.5μM EV primers & 0.5μM β actin primers } Using KCl buffer

Lane 7: Amplified products using 0.5μM EV primers & 0.2μM β actin primers

3.4 Positive Controls and Optimization of Their Annealing Temperatures

The positive controls used in this study were derived from viral isolates and cultures. These positive controls include EV71, Influenza A, Influenza B, Dengue 1, Dengue 2, Dengue 3, Dengue 4, Epstein-Barr virus and Cytomegalovirus. After the viral DNA and RNA had been extracted, they were amplified by using their respective virus primers before the primers were used for the screening of the clinical samples later on. Different annealing temperatures(55, 60, 65, 68^{0}C) for the amplification of each virus were optimized and the optimum annealing temperature in which the most intense band was generated is shown in the table 7 below:-

Table 7: Optimum annealing temperature of different viruses during PCR

Virus	Optimum Annealing Temperature (^{0}C)
Enteroviruses	55
Influenza A	60
Influenza B	55
Flaviviruses	65
Herpesviruses	46

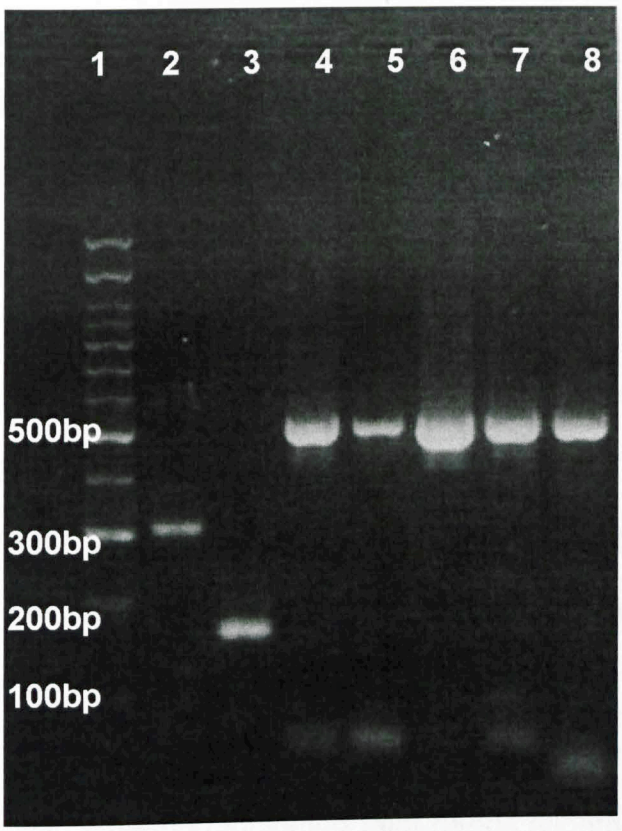


Figure 6

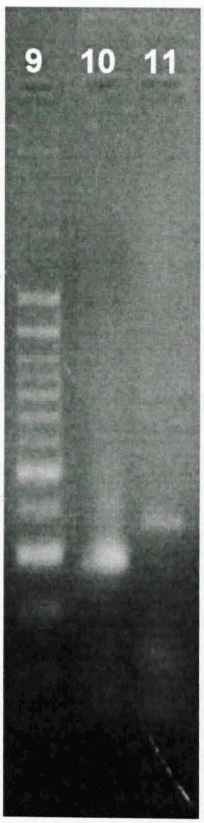


Figure 7

Figure 6 & 7: Gel electrophoresis of amplified positive controls

Lane 1: 100bp DNA ladder

Lane 2: Influenza A

Lane 3: Influenza B

Lane 4: Dengue Type 1
Lane 5: Dengue Type 2
Lane 6: Dengue Type 3
Lane 7: Dengue Type 4
} Flaviviruses

Lane 8: EV71 (Enterovirus)

Lane 9: 100bp DNA ladder

Lane10: Epstein-Barr virus
Lane 11: Cytomegalovirus
} Herpesviruses

3.5 Interpretation of Gel Electrophoresis of Clinical Samples

The PCR products of the clinical samples were electrophoresed in 1.5% w/v agarose gels at 40mA for 45 minutes. At the end of electrophoresis, the gels were viewed under a UV transilluminator.

Before the screening for the different viruses, all samples were screened with β actin genes to ensure the internal control was amplified. The lower half of each of the gel photos showed the successful amplification of the β actin genes.

For the screening of Enteroviruses (EV) using EV degenerate primers which can amplify all viruses from the EV genus, all cases showed negative results (no bands on targeted region) except 4 inflammatory myopathy cases where multiple bands with smearing including faint bands slightly below the targeted region were observed on the gel which suggested the cases to be suspected positive.

For the screening of Influenza A virus, all samples were negative except 3 inflammatory myopathy cases where multiple bands were observed on the gel.

As for the screening of Influenza B virus, all the cases were also negative as no targeted band was noted.

Screening for the flaviviruses family using degenerate primers clearly showed a negative association between the two muscle diseases (acute rhabdomyolysis and inflammatory myopathy) with flaviviruses infection. No bands were observed and non specific bands were also absent.

Lastly, screening for herpesviruses family which can amplify viruses from the Herpesvirus family also demonstrated negative association between the muscle diseases and herpes viruses.

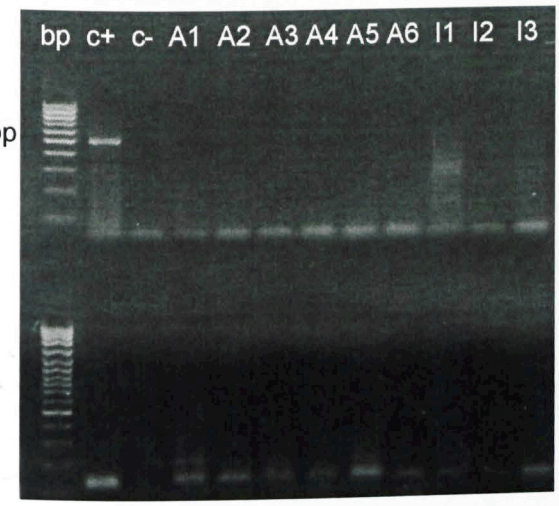


Figure 8

Figure 9

Figure 8 & 9: Screening for EV and amplification of β actin genes

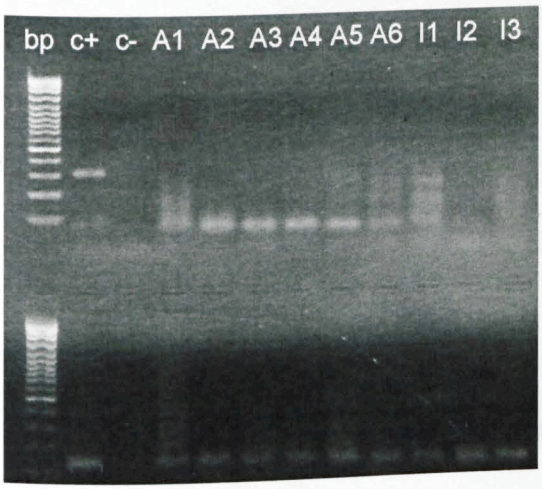


Figure 10

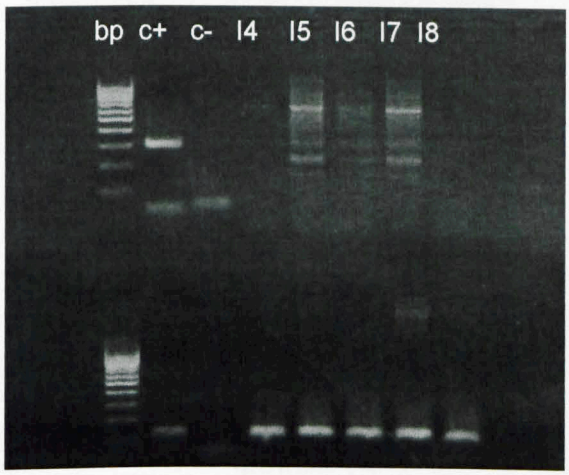


Figure 11

Figure 10 & 11: Screening for Influenza A and amplification of β actin genes

Legend:

bp : 100 bp DNA ladder

C+: Positive Control

C- : Negative Control

A : Acute rhabdomyolysis case

I : Inflammatory myopathy case

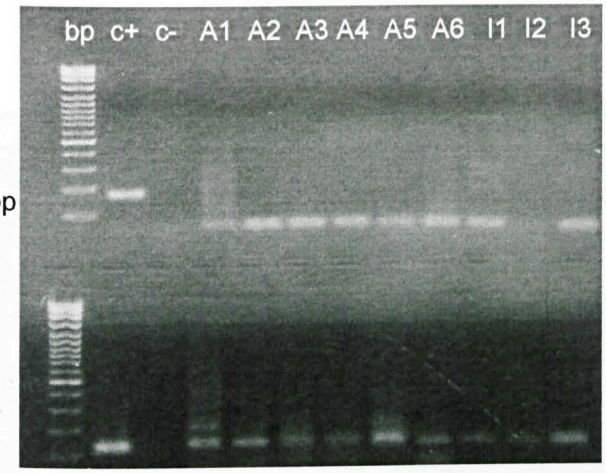


Figure 12

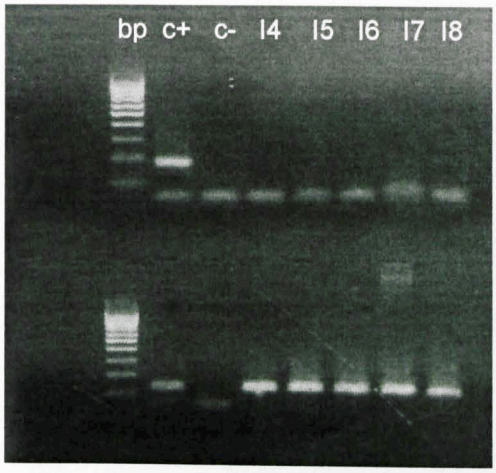


Figure 13

Figure 12 & 13: Screening for Influenza B and amplification of β actin genes

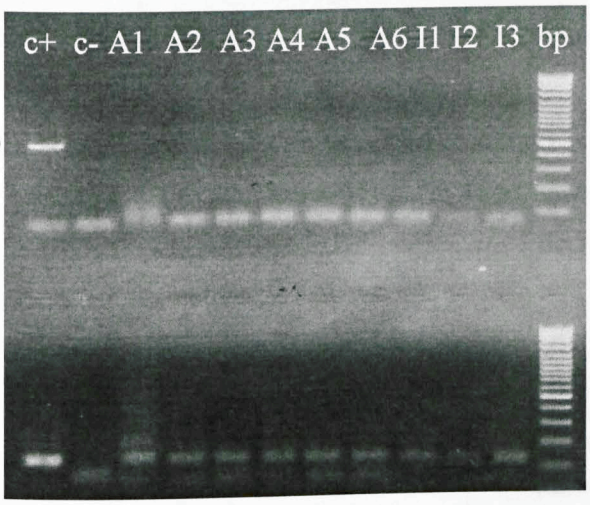


Figure 14

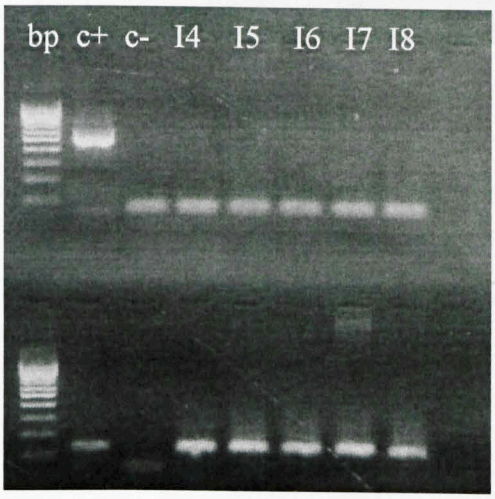


Figure 15

Figure 14 & 15: Screening for Flavivirus and amplification of β actin genes

Legend:

bp : 100 bp DNA ladder
C+: Positive Control
C- : Negative Control
A : Acute rhabdomyolysis case
I : Inflammatory myopathy case

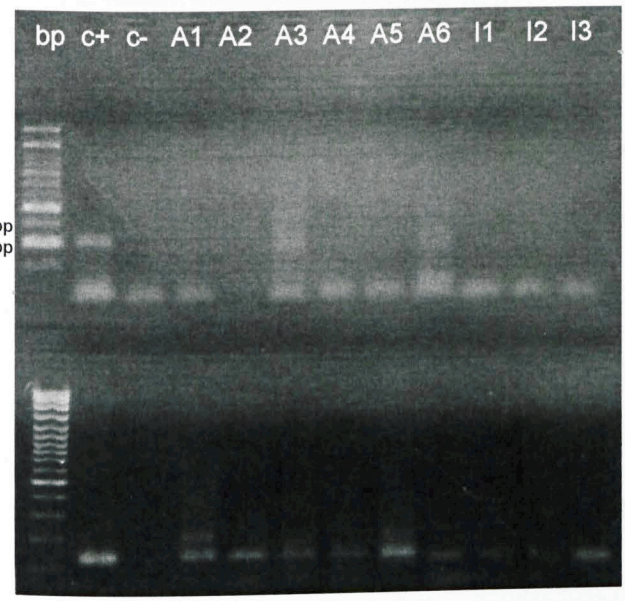


Figure 16

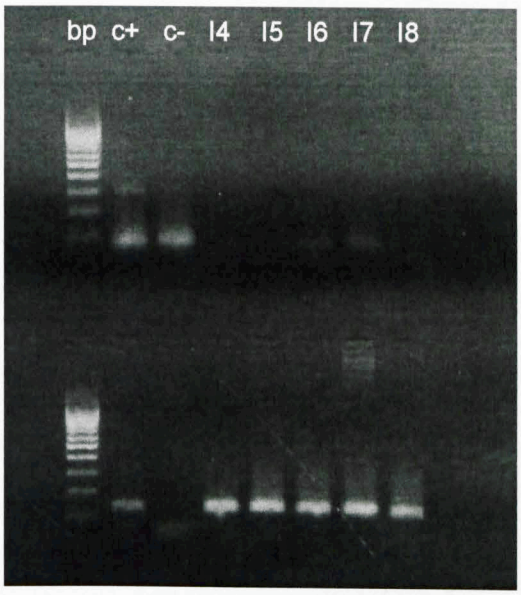


Figure 17

Figure 16 & 17: Screening for Herpesvirus and amplification of β actin genes

Legend:

bp : 100 bp DNA ladder

C+: Positive Control

C- : Negative Control

A : Acute rhabdomyolysis case

I : Inflammatory myopathy case

3.6 Immunohistochemistry (IHC)

IHC was performed as a rapid, simple and additional tool to confirm the PCR suggestive of the EV positive for the inflammatory myopathy case (P03/3) as shown before. EV71 infected vero cells were used as positive control while mock infected cells were used as negative control. EV polyclonal antibody which can cross react with all the viruses from the Enterovirus group was used as primary antibody while swine anti rabbit was used as the secondary antibody. Results showed that this case was indeed negative as no immunoreactivity was observed in the suspected sections.

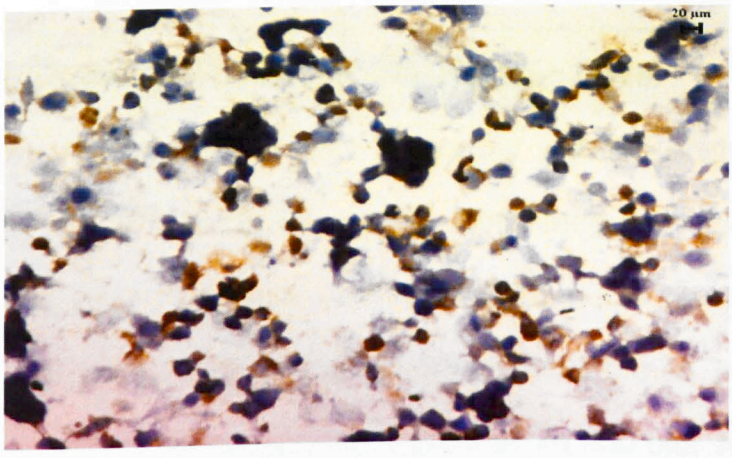


Figure 18: IHC of EV positive control (EV71 infected vero cells)

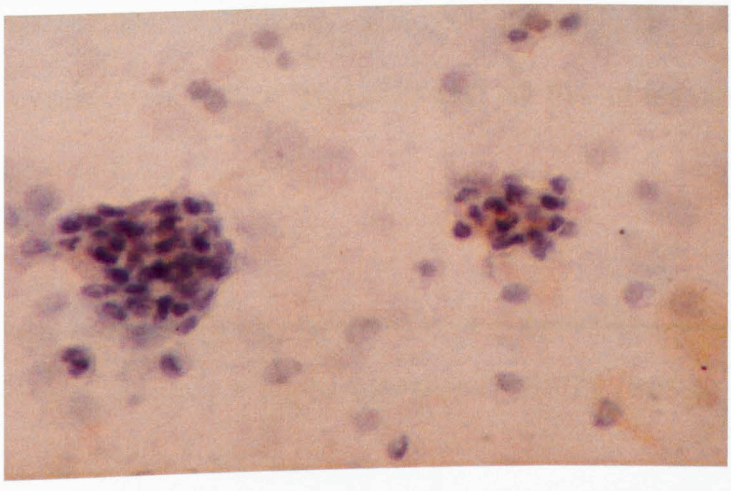


Figure 19: IHC of EV negative control (Mock infected vero cells)

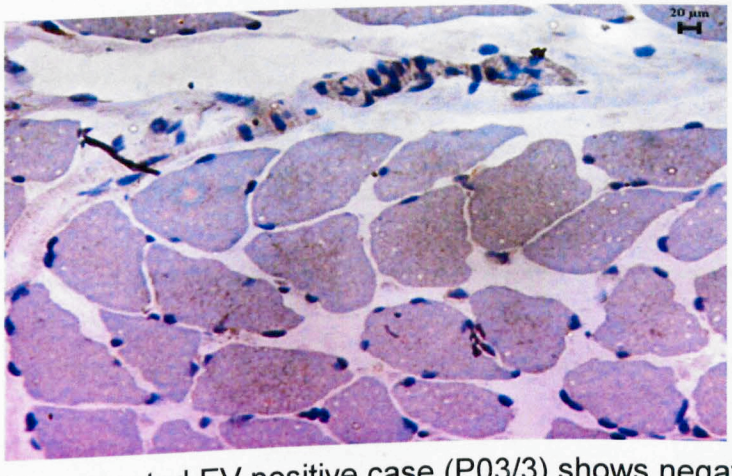


Figure 20: IHC of 1 suspected EV positive case (P03/3) shows negative staining

3.7 DNA Sequencing of One Suspected EV Positive Case

One inflammatory myopathy case suspected with EV positive was sent for DNA sequencing. Result was obtained in the chromatography form *(Appendix D)* and the sequencing data was blasted with nucleotide-nucleotide BLAST available in the net (Altschul *et al.*, 1997). The amplified band turned out to be human genomic DNA (Chromosome 11) with the highest score and bits. None of the listed homology sequences matched any of the viral genome from the EV genus had suggested that nonspecific amplification of human genomic DNA was obtained. We conclude that the case was indeed negative and all the other cases present with multiple bands were also non specific.

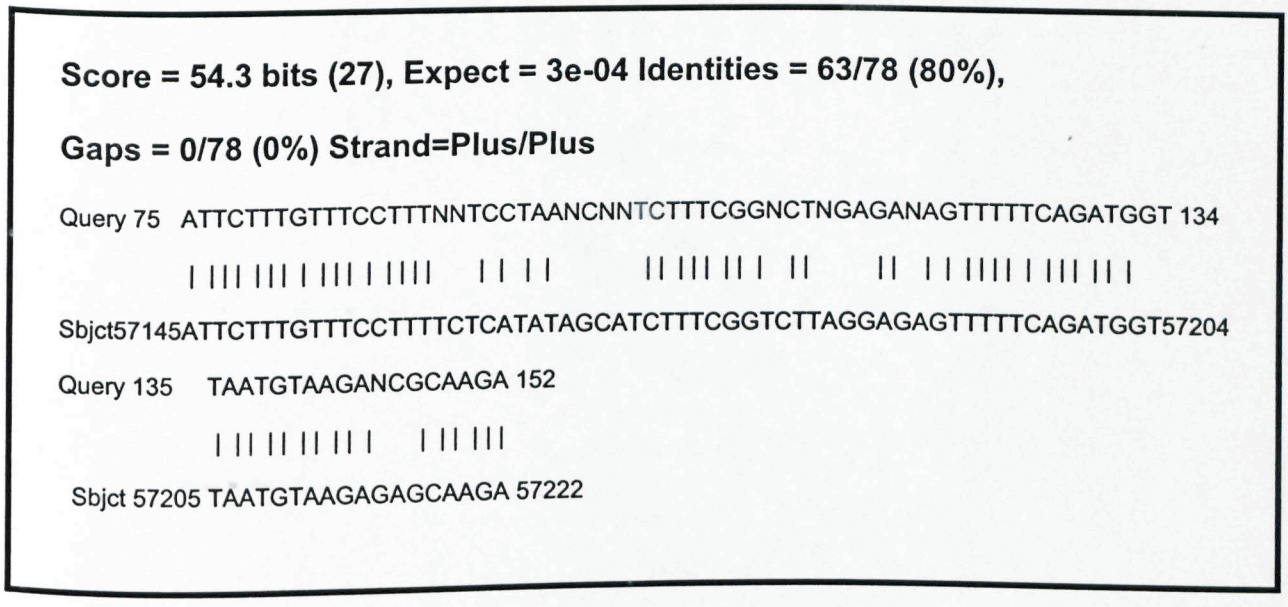


Figure 21: DNA sequencing result of one suspected EV positive case

CHAPTER 4

DISCUSSION

DISCUSSION

4.1 Availability of Samples and References

In this study, the association between acute rhabdomyolysis and inflammatory myopathy with viral infection was investigated by using archival frozen muscle biopsy samples. There were only 5 cases of acute rhabdomyolysis and 8 cases of inflammatory myopathy ranging from the year 1994 to 2006 identified in this study. The diagnosis of these cases have been confirmed previously by the clinicians based on the anatomic pathology report which were retrieved from the muscle biopsy files from the Department of Pathology, University of Malaya.

The sample size for both acute rhabdomyolysis and inflammatory myopathy is small because these muscle diseases are rare and uncommon in Malaysia. Besides, no previous studies had been performed in Malaysia to investigate the association of these 2 muscular diseases with viral infection. In addition, recent literatures or reports regarding this were very scarce and limited as well. The scarcity of the reports of rhabdomyolysis associated with viral infection in the mainstream medical literature had suggested that it is a very rare complication. Besides, many cases may have gone under reported and under recognized (Davis and Bourke, 2004)

The 13 clinical samples selected in this study possess features such as necrosis and degeneration with surrounding inflammatory cells including neutrophils, macrophages and lymphocytes that suggested a possibility of viral infection based on their respective anatomic pathology reports. With this, it had

prompted the interest to investigate the association of viral infection with acute rhabdomyolysis and inflammatory myopathy.

4.2 Methodologies

To investigate the association between viral infection and rhabdomyosis, if any, the molecular technique using RT-PCR was employed. Prior to the RT-PCR, two methods of in house RNA extraction either with or without the incorporation of Proteinase K in tissue digestion were being compared. The results showed that combination of Proteinase K and Trizol ® Reagent was able to give higher yield of RNA and comparable purity with only the Trizol ® Reagent alone.

This result denoted that combination of Proteinase K and Trizol ® Reagent is a better method to be employed in total RNA extraction.

The distinctness of these two methods in terms of RNA yield and purity may be due to the incomplete digestion of the muscle tissue purely by using TRIzol ® Reagent. As the muscle tissues of the mice which was added with TRIzol ® Reagent and proteinase K were incubated for 1 hour in the water bath at 55°C, it allowed complete digestion of the muscle tissues and thus yield higher and purer total RNA.

RT-PCR is chosen as the primary method to be used in this study to detect the presence of viral genome in the muscle biopsies as it permits the analysis of samples from as little as one cell in the same experiment (Bustin, 2000). It is the most sensitive and the most flexible quantification method compared to other RNA quantification methods such as Northern blotting and Rnase protection assay (Wang and Brown, 1999). Besides, it was reported that PCR is a rapid detection

method for virus and it has more sensitivity than standard tissue culture, immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) (Poddar, 2001). Specificity, sensitivity and reproducibility are very important characteristics for clinical diagnostic uses (Bustin, 2000).

Three types of RT primers were compared using the RNA of EV71 infected limb muscle of the mice to compare its sensitivity. RT step can be primed using three types of primers which are gene specific primers, random hexamer and oligo dT primers.

Oligo dT which binds to the endogenous poly(A)$^+$ tails of mammalian mRNAs, can be used as a universal primer for conventional first strand cDNA synthesis (Sambrook and Russell, 2001).

Random hexamer, which are capable of priming cDNA synthesis at many points along RNA templates, generate fragmentary copies of the entire population of RNA molecules. They are useful when the target RNA is extremely long or contains a lot of secondary structure that cDNA synthesis cannot be efficiently primed by oligo dT or synthetic oligonucleotides (Sambrook and Russell, 2001).

Gene specific amplification is achieved by using sense and antisense primers targeted to sequences in the cDNA. In most cases it is best to generate a first strand of cDNA that is as long as possible and contains a high proportion of molecules complementary to the target RNA. Gene specific primers that bind to the 3'-ultrastrand region of the target mRNA is thus able to generate almost a full length of cDNA (Sambrook and Russell, 2001).

The use of gene specific primers could decrease background priming, wheras the use of random hexamer and oligo dT primers maximize the number of

mRNA that can be analyzed from a small sample of RNA (Bustin, 2000). The result from gel electrophoresis was in agreement with the previous findings (Radhakrishnan *et al.*, 1999; Bustin, 2000) as both random hexamer and gene specific primers had managed to produce strong bands. This is further supported by the fact that random hexamer are capable of priming cDNA synthesis at many points along RNA templates and generate fragmentary copies of RNA (Sambrook and Russell, 2001). Besides, random hexamer can anneal at many points compared to gene specific primers and oligo dT primers, thereby synthesizing more templates of cDNA (Radhakrishnan *et al.*, 1999). Thus, random hexamer had chosen to be the preferable RT primer to be used in the RT step on the subsequent screening of the 13 clinical samples.

2 different buffers (Taq buffer with $(NH_2)SO_4$ and KCl buffer) were used in the comparative study using both EV degenerate primers and β actin primers and RNA from EV71 infected limb muscle of the mice in duplex PCR. It was found that KCl buffer was more consistent in each PCR reaction. This had been supported by a previous finding in which KCl had been suggested to demonstrate certain advantages such as it is less complex and easier to adjust and optimize, it requires less dNTP during PCR compared to other buffers which will in turn enhance the fidelity of Taq DNA Polymerase as more Mg^{2+} ion will react with Taq DNA Polymerase (Henegariu *et al.*, 1997).

Internal control is included before the screening of viral genome is done to ensure that there's no presence of inhibition in the clinical samples which might suppress the subsequent amplification of the viral genome if there is any.

Beta actin mRNA is expressed at moderately abundant levels in most cell types and encodes a ubiquitous cytoskeleton protein. It was one of the first RNA to be used as an internal standard in RT-PCR assays. However, extra caution should not be underestimate with the use of beta-actin as internal control in gene expression study as its transcription level has been reported to be affected by the experimental design (Bustin, 2000).

In this study, the optimization of the annealing temperatures for most of the positive controls (Enterovirus 71, Influenza A, Influenza B, Dengue 1-4) had also been performed. Four temperatures (55, 60, 65 and 68^{0}C) were being attempted to determine the most optimum annealing temperature for each of the positive controls based on their band intensity on AGE. The rest of the PCR condition in this optimization study was an in house adapted condition.

4.3 Results

Almost all cases were negative except 4 inflammatory myopathy cases which were suspected to be EV positive and another 3 inflammatory myopathy cases which were suspected to be Influenza A positive. However, these results were not convincing enough as there were present of multiple bands on the targeted region which signified that the results were not specific. In order to confirm the results, one of the inflammatory myopathy cases suspected to be EV positive was tested with IHC using EV polyclonal antibody and sent for DNA sequencing at the same time.

DNA sequencing result showed that the band obtained at the targeted region was actually human genomic DNA (human chromosome 11) instead of any viral

genome from the Enterovirus genus. This may be due to the reason that some genomic DNA had been accidentally extracted together with the RNA during RNA extraction. Other than that, there was no incorporation of DNAase which might cause DNA to have a tendency to be extracted together with RNA.

4.4 Other Possible Etiologies of Acute Rhabdomyolysis and Inflammatory Myopathy

In this study, no viral infection was shown to be positively associated with both acute rhabomyolysis and inflammatory myopathy. The low prevalence of these two muscular diseases, and many other etiologies known to be associated or caused rhabdomyolysis and inflammatory myopathy that have not be investigated were the main obstacles encountered in this study. Both diseases could have been caused by a vast variety and diverse causes which had been reported widely.

Among the known causes of rhabdomyolysis and inflammatory myopathy are direct muscle injury, excessive physical exertion, hereditary enzyme defects and other medical causes such as drugs and toxins, electrolyte imbalances, metabolite defects, or infection (Pesik and Otten, 1996; Allison and Bedsole, 2000). However, most cases of rhabdomyolysis reported in the literatures were due to adverse effect of drugs and toxins (Pesik and Otten, 1996).

Acute rhabdomyolysis and inflammatory myopathy as a result of infection could be due to bacterial, viral, fungal or parasitic infection. Only viral infection is reviewed in this study. However, according to Pesik et al., viral infection in rhabdomyolysis is a complication that is not often considered (Pesik and Otten, 1996). Besides, it has been reported that acute renal failure which is probably due

to rhabdomyolysis had occurred in 57% of the patients with a bacterial cause as compared to only 34% of those with a viral cause (Guis *et al.*, 2005). In addition, it has also been suggested that rhabdomyolysis due to nonspecific effects could be obtained with either bacterial or viral infection since both have been associated with these diseases (Berlin *et al.*, 1974). As for inflammatory myopathy, it is very uncommon to be associated with viral infection. This is also partly due to the scarcity and insufficiency of the cases reported in the literatures. Though the association of IBM and mumps virus had been proposed, nevertheless uses of immunological techniques had failed to prove the association (Nishino *et al.*, 1989; Kallajoki *et al.*, 1991).

Despite multiple etiologies had been reported to be associated with rhabdomyolysis and inflammatory myopathy, only viral infection had been investigated in this study. This is due to the reason that patients' histories and anatomic pathology reports of these 13 cases had reported inflammation and presence of phagocytes and mononuclear cells with a sudden onset of illness followed by rapid recovery within a short period of time which had suggested a higher possibility of viral infection.

4.5 Limitations of the Study

The most considerable limitation encountered in this study is the insufficiency and scarcity of the clinical samples. Acute rhabdomyolysis is a very rare muscle disease in Malaysia, with only 5 cases reported in 12 years time (from 1994 to 2006) in which the etiologies remain to be investigated. Inflammatory myopathy, though considered rare as well, is slightly more prevalent compared to

acute rhabdomyolysis. However, the potential cases to be investigated in this study whereby the causes were probably due to viral infection were very scanty as well.

Besides, literatures regarding the association of acute rhabdomyolysis and inflammatory myopathy with viral infection were barely sufficient and adequate. This may probably due to under-reporting and under-recognition of such a complication by physician (Lim and Goh, 2005). Besides, such similar studies had never been performed in a Malaysian population. As a result, there were not much laboratory investigation methodologies to be referred to in this study.

All the 13 cases of clinical samples used in this study were in the form of muscle biopsy. Most cases reported in the literatures were immediate case reports following the admission of patients into the hospital and the samples were mostly in the form of fresh patient serum. In most studies, serological techniques were used to access the virus using patient's serum samples which were more effective in detecting the virus. However in this study, most of the samples were many years ago and there was no way of obtaining the patient serum samples.

Due to limited time frame in this study, only the association of viral infection with acute rhabdomyolysis and inflammatory myopathy had managed to be reviewed. As acute rhabdomyolysis and inflammatory myopathy could also due to bacterial, fungal or parasitic infection (Rendt, 2001; Allison and Bedsole, 2003; Criddle, 2003), investigation of these muscle diseases with other microorganism infections should also be included in this study.

4.6 Future Studies

Future studies should also investigate the association of acute rhabdomyolysis and inflammatory myopathy with bacterial infection, fungal infection and parasitic infection. This is to ensure that the investigation of these three muscular diseases (acute rhabdomyolysis, polymyositis and inclusion body myositis) with the possibility of microorganism infections is more complete and accurate. Other types of microorganism infections should also be inspected in the investigation before a solid conclusion regarding this association is made.

Besides, the association of these muscular diseases (acute rhabodmyolysis and inflammatory myopathies) with drugs and toxins should also be investigated as this was the most common etiologies reported in the mainstream literatures.

CHAPTER 5

CONCLUSIONS

CONCLUSIONS

Molecular screening for the presence of viral genomes in all the 13 available frozen muscle biopsy samples with five different types of viruses of four families (Enteroviruses, Flaviviruses, Herpesviruses, Influenza A and Influenza B) had shown no association between viral infection and the muscular diseases (acute rhabdomyolysis, polymyositis and inclusion body myositis).

More clinical samples should be included in future in order to investigate this association as a wider coverage and larger sample size from the Malaysian population is very important in ensuring the validity and potency of this study.

APPENDIX

APPENDIX A

PREPARATION OF REAGENT FOR TOTAL RNA AND RNA EXTRACTION:

1. Tissues lysis buffer

a. 1M Tris (pH8.0) (Tris-hydroxymethy) amonomethane, 1L

- Tris base 121.1g
- Double distilled water ..... to 1000ml
- pH adjust with concentrated HCl to pH 7.5
- Autoclave at 121^0C for 15 minutes

b. 0.5M EDTA (pH8.0), 1L

- EDTA.$2H_2O$ 186.1g
- Double distilled water ..... to 1000ml
- Adjust pH with 5M NaOH to pH8.0

c. 5M NaCl, 1L

- NaCl 292.2g
- Double distilled water ..... to 1000ml
- Shake vigourously until dissolve

d. 10% Sodium dodecyl sulphate (SDS), 100ml

- SDS 10g
- Double distilled water ..... to 100ml
- Filter sterilized

e. 5M NaOH, 1L

- NaOH 200g
- Double distilled water ..... to 1000ml
- Shake vigorously until dissolved

Working solution for digestion buffer, pH8.4:-

Solution	Final concentration
TRIS-HCL	10mM
EDTA	100mM
NaCl	100mM
SDS	0.5%

Note: The working solution is adjusted to pH8.4 using 5M NaOH.

2. Digestion buffer with 20 μg/ml proteinase K

- Digestion buffer, pH 7.8 ..... 1.99 ml
- Proteinase K, 20 mg/ml..... 10 μl

Solutions were mixed prior to use.

3. Sodium acetate, 3M

- Sodium acetate ..... 40.81 g
- Double distilled water ..... 80 ml

Sodium acetate was dissolved in dH_2O and pH was adjusted to 5.2 with glacial acetic acid before the volume was topped up to 100ml with dH_2O.

APPENDIX B

PREPARTION OF REAGENT FOR AGAROSE GEL ELECTROPHORESIS:

1. Tris-Borate EDTA buffer (TBE buffer), 5X

- Tris base ..... 54.5 g
- Boric acid ..... 27.81 g
- EDTA ..... 1.86 g
- Add double distilled water to a final volume of 1000 ml.

All the chemicals were dissolved in a dH_2O to final volume of 1liter. 1 X TBE working buffer was prepared by diluting with dH_2O and pH adjusted to 8.3.

2. Ethidium bromide (EtBr) 10 mg/ml

- EtBr powder ..... 0.2 g
- Add double distilled water to a final volume of 20 ml.

EtBr powder was dissolved in dH_2O by stirring and stored in the dark.

Note: EtBr is mutagenic and must be handled carefully in a fume hood cupboard.

3. Loading dye

- Bromophenol blue/xylene cyanol (loading dye) 10ml
- 0.25% Bromophenol blue 0.025g
- 0.25% xylene cyanol 0.025g
- 15% ficoll 400 ..... 1.5g
- Double distilled water 10ml

Filter and store at room temperature.

APPENDIX C

PREPARATION OF REAGENT FOR IMMUNOHISTOCHEMISTRY (IHC)

1. Tris buffered Saline (TBS), pH 7.6

- Tris base ..... 6.05 g
- NaCl ..... 80.0 g
- 1 N HCl ..... 38 ml
- dH_2O ..... 9 liter

Tris base and NaCl were dissolved in 9 liter of dH_2O. 38 ml of HCl was added and the pH was adjusted to 7.6 by adding concentrated HCl before topped up to 10 liter with dH_2O.

2. Hydrogen peroxide (0.6%)

- 30% Hydrogen peroxidase ..... 1 ml
- Methanol ..... 50 ml

Hydrogen peroxide was aliquoted using pipette and diluted in methanol. The solution was prepared fresh for each staining procedure.

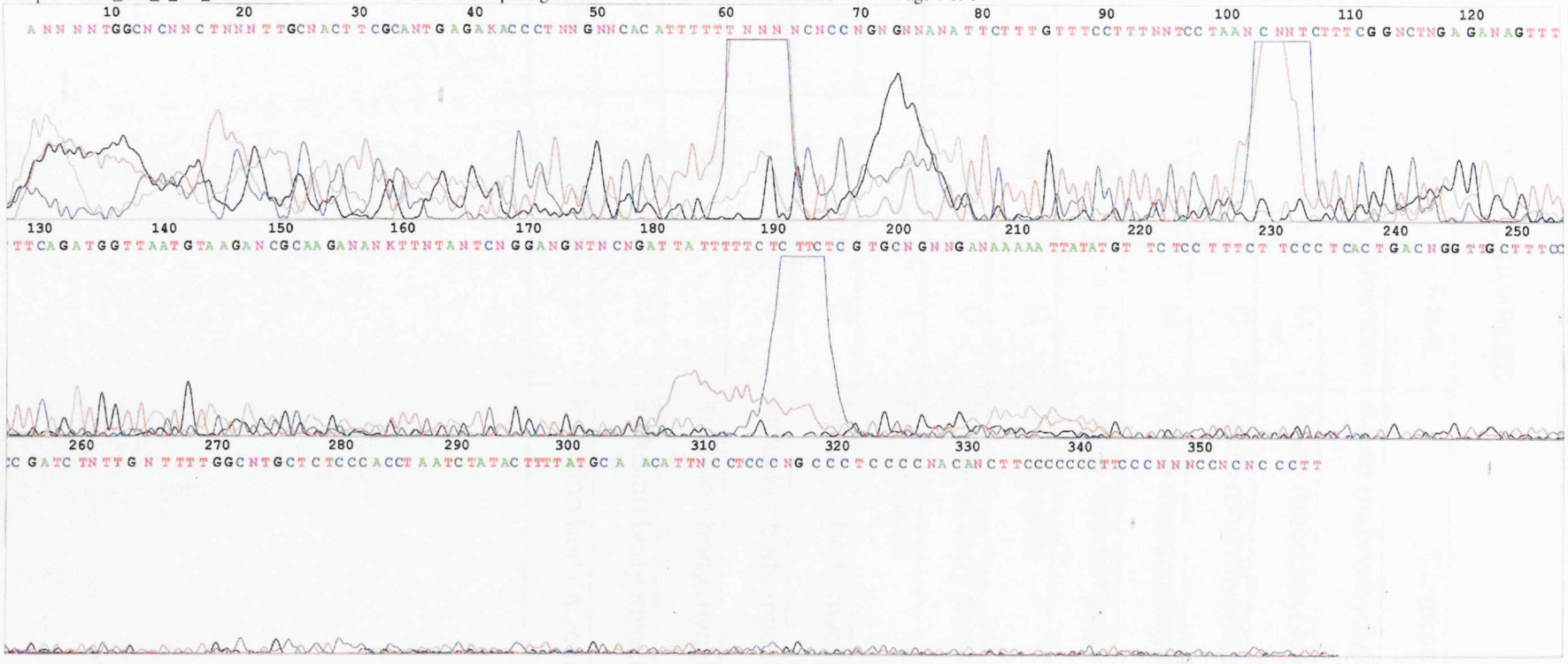
3. Diaminebenzidine tetrahydrochloride (DAB)

- DAB ..... 0.007 g
- Tris for DAB, pH 7.6 ..... 10 ml
- 30% hydrogen peroxide (H_2O_2) ..... 13 µl

DAB was dissolved in 10 ml tris buffer and 10 µl H_2O_2 was added to the prepared solution. The solution was prepared fresh, prior to use.

DNA SEQUENCING RESULT (P03/3)

File: 1st_BASE_28915_P03_3_EV_Positive.ab1 Run Ended: Jul 7, 2006, 11:17:51 Signal G:23 A:17 T:30 C:25 Comment:
Sample: 28915_P03_3_EV_Positive Lane: 9 Base spacing 13.13 356 bases in 4346 scans Page 1 of 1



APPENDIX E

CASE PROFILES OF 13 CLINICAL SAMPLES

Lab No.	Sex	Age	Race	Diagnosis
E94-650	Unknown	Unknown	Unknown	Acute rhabdomyolysis
G51-97	F	Unknown	M	Acute rhabdomyolysis
G3-99	F	23	C	Acute rabdomyolysis
G04-144	M	16	M	Acute rhabdomyolysis
P06-84	M	24	C	Acute rhabdomyolysis
G05-197	F	25	C	Polymyositis
P03-3	M	19	C	Inclusion body myositis
P03-319	M	21	I	Polymyositis
S00-4876	M	17	C	Inclusion body myositis
G01-101	M	19	C	Inclusion body myositis
S01-8654	F	51	M	Inclusion body myositis
G01-186	F	68	M	Inclusion body myositis
S01-6009	M	73	C	Inclusion body myositis

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EXTENDED ABSTRACT

INTRODUCTION

Rhabdomyolysis is a phenomenon whereby skeletal muscles undergo acute destruction and necrosis. It could produce nonspecific clinical syndrome that causes extravasation of toxic intracellular contents from the myocytes into the circulatory system (Criddle, 2003).

The syndromes of acute rhabdomyolysis comprises of myoglobinuria, generalized edema, muscle tenderness and weakness and also the progressive darkening of the urine (McDonnell, 2002). Myoglobinuria is often seen after viral infection (Dalakas, 2004).

Inflammatory myopathy is a form of muscle weakness which includes polymyositis, dermatomyositis and inclusion body myositis (IBM). In this study, only IBM and polymyositis will be studied. IBM is the commonest form of inflammatory muscle disease after the age of 50, with a 3:1 male predominance (Mikol and Engel, 2004). IBM may be sporadic or familial and may occur in early childhood (Carpenter and Karpati, 2001). On the other hand, polymyositis is best defined as a subacute myopathy that evolves over weeks to months, affects adults but rarely children (Dalakas and Hohlfeld, 2003).

To date, there are multiple etiologies that are known to be associated with rhabdomyolysis and IBM. Allison and Bedsole in year 2003 had classified the etiologies of rhabdomyolysis into four broad categories which include trauma or direct muscle injury, excessive muscle activity, hereditary muscle enzyme defects and other medical causes such as drugs and toxins, muscle hypoxia, metabolic

and endocrine disorders, viral and bacterial infections, temperature alterations and miscellaneous rare causes (Allison and Bedsole, 2003).

The association of rhabdomyolysis and viral infection had been reported in numerous publications previously. Among the viruses known to be associated with rhabdomyolysis include Dengue virus, West Nile virus, Enteroviruses (Coxsackie viruses A&B and ECHO virus), Influenza A, Influenza B and Herpes viruses (Herpes-simplex virus, Epstein-Barr virus and Cytomegalovirus) and Human Immunodeficiency Virus (HIV) (Chariot *et al.*, 1994, Pesik and Otten, 1996, Josselson *et al.*, 1980, Shenouda and Hatch, 1976, Davis and Bourke, 2004, Montgomery *et al.*, 2005, Konrad *et al.*, 1993). According to several reports, the association of IBM with viruses has not been documented so far. Studies to detect paramyxovirus on the muscle biopsies of patients with IBM using immunocytochemistry and in-situ hybridization had appeared to be negative (Kallajoki *et al.,* 1991, Nishino *et al.,* 1989). As for polymyositis, though several viruses (coxsackieviruses, influenza, parvoviruses, paramyxoviruses, cytomegaloviruses, Epstein-Barr virus) had been reported to be indirectly associated with myositis, PCR had not been able to amplify viral genome from the muscle tissues of these patients (Dalakas and Hohlfeld, 2003).

The overall objective of this study is to detect the presence of viral genomes (Influenza A, Influenza B, Flaviviruses, Enteroviruses and Herpesviruses), if any, in the frozen muscle tissue of patients diagnosed with acute rhabdomyolysis and inflammatory myopathy.

MATERIALS AND METHODS

• Clinical Samples

The clinical samples used in this study consisted of frozen skeletal muscle biopsy tissues embedded with OCT compound and stored at -80°C. There were 13 clinical samples in this study, 5 samples of acute rhabdomyolysis and 8 samples of sporadic IBM.

• Positive Controls

The positive controls used in this study were Dengue virus (serotype 1-4), Influenza A, Influenza B, Enterovirus 71 (EV71), Cytomegalovirus (CMV) and Epstein-Barr virus (EBV). These virus isolates were obtained in the form of viral cultures except EV71 which was obtained from EV71 infected limb muscle of the mice. RNA viruses were extracted by using Viral RNA extraction kit while DNA viruses were extracted by using QIAamp ® DNA Mini Kit.

• Comparison of 2 RNA Extraction Methods

One of the RNA extraction methods was performed by digesting the muscle tissue by using TRIzol ® Reagent while the other method was performed using Proteinase K solution followed by incubation in the water bath at 55°C for 1-2 hours before the subsequent addition of TRIzol ® Reagent. These 2 methods were compared in terms of RNA yield and RNA purity to determine the better method to be employed on the clinical samples.

• Extraction of Total DNA

Extraction of DNA was performed directly on the 13 clinical samples by using phenol-chloroform extraction followed by overnight precipitation with sodium acetate and absolute ethanol.

• Comparison of 3 Reverse Transcriptase (RT) Priming Steps

3 types of RT primers were being compared: gene specific primer, random hexamer and oligo dT primer. The reaction mixture for RT of each priming method consisted of 1X reaction buffer, 0.5mM of dNTP, 10U of Rnase inhibitor, 10U of RT-AMV, 5.0μM of Oligo dT primers, 2.5μM of gene specific primers or 50μg/ml of random hexamer, 1μg of RNA from EV71 infected limb muscle of the mice and double distilled Mili Q water. The cycling condition for RT steps is 42°C for 60 minutes, 95°C for 2 minutes and hold at 4°C.

•Optimization of 2 Different PCR buffers and Different Concentration of Primers in Duplex PCR

2 different PCR buffers (Taq buffer with $(NH_2)SO_4$ and KCl buffer) were used in the optimization study. These 2 buffers were compared in terms of the consistency and sensitivity during each PCR reaction. Besides, different concentrations of primers (β actin and EV primers) were also attempted in duplex PCR to determine which combination or proportion of the two primer concentrations could produce the most optimum result.

• Internal Control

β actin (housekeeping gene) is used as an internal control. PCR condition for internal control is: 25μl of total reaction consists of one time normal PCR buffer without $MgCl_2$, 3mM $MgCl_2$, 0.2mM dNTP mix, 0.05Unit/μl Taq Polymerase, 0.5μM β actin forward primer, 0.5μM β actin reverse primer, in house prepared double distilled Mili-Q Water and 2μl of cDNA template. The cycling condition for internal control is initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C

for 50 seconds, 55^{0}C for 1 minute, 72^{0}C for 1 minute and final extension at 72^{0}C for 10 minutes.

• Polymerase Chain Reaction (PCR)

PCR was carried out in the following condition: 25µl of total reaction consists of one time normal PCR buffer without $MgCl_2$, 3mM $MgCl_2$, 0.2mM dNTP mix, 0.05Unit/µl Taq Polymerase, 0.5µM forward primer, 0.5 µM reverse primer, in house prepared double distilled Mili-Q Water and 2µl of cDNA template. The general cycling condition for each viral genome is 94^{0}C for 5 minutes (initial denaturation), 94^{0}C for 50 seconds (denaturation), annealing temperature depends on the optimization of respective positive controls, 72^{0}C for 1 minute followed by final extension at 72^{0}C for 10 minutes.

• Agarose Gel Electrophoresis and Photography

The amplified DNA products were electrophoresed in a 2.0% agarose gel. The gel will then be visualized by using UV transilluminator.

• Purification of the Amplified DNA Products and DNA Sequencing

The sample suspected to be positive (presence of faint light band on the gel) was purified by Gene $^{\surd}$All ™ DNA Purification Kit and sent for DNA sequencing.

• Immunohistochemistry (IHC)

Besides DNA sequencing, one of the suspected EV positive case was also investigated by IHC, using EV polyclonal antibody which will cross react with all species of viruses form the EV family.

RESULTS

• Comparison of 2 RNA Extraction Method

Of the 2 RNA extraction methods above, it was found that using proteinase K solution followed by TRIzol ® reagent had yielded higher concentration of RNA. Thus this method is employed on the RNA extraction of clinical samples.

• Comparison of 3 Different RT Priming Steps

Random hexamer was chosen as the preferable RT primers to be used on the clinical samples as it has higher sensitivity to prime the RNA.

• Optimization of Duplex PCR Using Different Buffers and Different Concentration of Primers

From duplex PCR using β actin and EV primers, it showed that different combinations or proportion of primer concentrations did not really influence the migration of the DNA as well as the intensity of the bands. However, KCl buffer has the ability to generate stronger and more intense bands compared to Taq buffer with $(NH_2)SO_4$. Thus, KCl buffer had chosen to be used in the subsequent amplification of the clinical samples.

• Positive Controls and Optimization of their Annealing Temperature

The optimum annealing temperature is shown in the table below:-

Table 3: Optimum annealing temperature of different viruses during PCR

Viruses	Optimum Annealing Temperature (^{0}C)
Enteroviruses	55
Influenza A	60
Influenza B	55
Flaviviruses	65
Herpesviruses	46

• Interpretation of Gel Electrophoresis of Clinical Samples

For the screening of Enteroviruses, all cases showed negative results except four inflammatory myopathy cases where faint bands with smear were observed.

For the screening of Influenza A virus, all were negative except three inflammatory myopathy cases where multiple bands were observed on the gel including a faint and light band on the targeted region.

Screening of all clinical samples with Influenza B virus primers were negative.

Screening for the flaviviruses and herpes viruses were also negative.

• DNA Sequencing of 1 Suspected EV Positive Case

After blasting with nucleotide-nucleotide BLAST, the sequencing result showed that the sequence was similar to a human genomic DNA rather than any

viruses from the Enterovirus group. This had concluded that the case was negative.

- **Immunohistochemistry (IHC)**

IHC showed negative result (absence of viral genome on the tissue section).

DISCUSSIONS

The sample size for acute rhabdomyolysis and inflammatory myopathy is small because these muscle diseases are rare and uncommon in Malaysia. Proteinase K solution followed by TRIzol ® Reagent had chosen to be the RNA extraction method to be employed on clinical samples as this method is able to digest the muscle tissue more completely in order to yield higher concentration of RNA. Besides, random hexamer had chosen to become the preferable RT primer to be used in RT priming step for the 13 clinical samples as it is capable of priming cDNA synthesis at many points along RNA templates and generate fragmentary copies of RNA (Sambrook and Russell, 2001). Internal control is included before the screening of viral genome is done to ensure that there is no presence of inhibition in the clinical samples which might suppress the subsequent amplification of the viral genome if it is present in the tissue. β actin is used as an internal control in this study as its mRNA is expressed at moderately abundant levels in most cell types and encodes a ubiquitous cytoskeleton protein (Bustin, 2000). Overall, screening of all the 13 clinical samples were negative though there were present of multiple bands which may be due to non specific flanking of the virus primers at any point which could possess the same few initial sequence as the viral genome. Gene specific primers had concluded the result to be true negative as the gel

electrophoresis result showed smearing instead of a specific band at the targeted region. DNA sequencing result of one of the inflammatory myopathy cases suspected to be EV positive was proven to be human genomic DNA instead of viral genome. Though acute rhabdomyolysis and inflammatory myopathy due to infection could be due to bacterial, viral, fungal or parasitic infection besides many other diverse etiologies, only viral infection is reviewed in this study. Future studies using more cases should be done to investigate possible viral etiology and association of acute rhabdomyolysis and inflammatory myopathy with viral infection.

CONCLUSIONS

Acute rhabdomyolysis and inflammatory myopathy are very rare muscular disorder that present in the Malaysian population. From the screening of these limited samples, there are no association of viral infection with acute rhabdomyolysis and inflammatory myopathy.